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(54) **Noninvasive vaccination through the skin**

Nichtinvasive Impfung durch die Haut

Vaccination non invasive à travers la peau

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WO-A-91/01146 **WO-A-92/04009**

- **PAUL A, CEVC G: "Non-invasive administration of protein antigens: transdermal immunization with bovine serum albumin in transfersomes" VACCINE RESEARCH, vol. 4, no. 3, 1995, pages 145-164, XP002107365**
- **PAUL A ET AL: "Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes" VACCINE, vol. 16, no. 2-3, 2 January 1998, page 188-195 XP004098622**
- **CEVC G: "Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery" CRITICAL REVIEWS IN THERAPEUTIC DRUG CARRIER SYSTEMS, vol. 13, no. 3-4, 1996, pages 257-388, XP002107366**

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Description

[0001] The present invention relates to novel vaccines for the non-invasive, transcutaneous administration of antigens associated with ultradeformable carriers, for the purpose of prophylactic or therapeutic vaccination. The vaccines comprise (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself, and (c) an antigen or an allergen. The invention further relates to methods for corresponding therapeutic or prophylactic vaccination of mammals.

[0002] Skin is the best accessible, but also the most difficult, entry into the body, due to the presence of the stratum corneum. This horny layer of the skin is an evolutionary optimised barrier which resembles the blood vessel wall, in that it comprises flaccid, tightly packed and laterally overlapping cells, whereby the basic cellular-tile motif in the stratum corneum is repeated 20-30 times. The intercellular contacts in the skin, moreover, are sealed with the densely packed and well organised blend of lipids. The stratum corneum therefore not only protects the organism from infections but also precludes an efficient uptake of antigens through the skin. This fact, which is advantageous from the point of allergy, prevented successful immunisation or vaccination through the intact skin to date.

[0003] The largest drugs on the market in any transdermal delivery device are smaller than 350 Da (Cevc, G. Drug delivery across the skin, Exp. Opin. Invest. Drugs (1997) 6: 1887-1937), as only such molecules can cross the tiny, self-sealing pores in the skin. The latter normally are less than 1 nm wide, when hydrophilic, or narrower, when hydrophobic. Organisms such as helminths therefore gain access into the body by penetrating the skin by using their biochemical machinery for the purpose of 'drilling holes' through the organ. Naturally occurring micro-lesions and shunts (such as pilosebaceous units) are available in the skin as well. However, they only cover up 0.1 % to 0.5 % of the skin surface and, consequently, do not contribute much to transcutaneous transport the fact notwithstanding that bacteria typically exploit such a route for a topical infection (Strange, P., Skov, L, Lisby, S., Nielsen, P. L., Baadsgard, O. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatoma. Arch. Dermatol. (1996) 132: 27-33.)

[0004] Only a few haptens exposed on the skin elicit a cutaneous immune response. This confirms that only sufficiently small molecules from a large load of the topically deposited haptens can find their way into the skin in an appreciable quantity. Such haptens then first irritate the organ and finally may cause hypersensitivity and contact dermatitis (Kondo, S., Sauder, D.N. Epidermal cytokines in allergic contact dermatitis. J. Am. Acad. Dermatol. (1995) 33: 786-800; Nasir, A., Gaspari, A. A. Contact dermatitis. Clinical perspectives and basic mechanisms. Clin. Rev. Allergy and Immunol. (1996) 14: 151-184). The problem is most serious with the low molecular weight chemicals or with the pharmaceuticals combined with skin irritants, such as skin permeation enhancers (Cevc,1997, *op. cit.*). Large molecules seldom are allergenic on the skin, owing to their limited ability to cross the barrier. A Th2 response to a highly immunogenic ovalbumin (Wang, L.-F., Lin, J.-Y., Hsieh, K.-H., Lin, R.-H. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with IgE production in mice. J. Immunol. (1996) 156: 4079-4082.) or to Cholera toxin (Glenn, G. M., Rao, M. Matyas, (1998) 391: 851; Glenn, G.M., Scharton-Karsten T, Vasell R, Mallet C.P., Hale T.L. and Alving C.R. Transcutaneous Immunization with Cholera toxin Protects Mice Against Lethal Mucosal Toxin Challenge . J. Immunol (1998) 161: 3211-3214.) was possible only after an epicutaneous exposure to a large amount of such proteins and was fairly weak. Moreover, the stratum corneum elimination from the skin was a prerequisite for producing detectable quantities of the specific antibodies against adenoviruses encoding the human carcinoembryonic antigen or human GM-CSF gene in 96 % or 43 %, respectively, of epicutaneously treated C57BL/6 mice (Deng, H., Qun, L., Khavari, P. A. Sustainable cutaneous gene delivery. Nature Biotechnology (1997) 15: 1388-1390.).

[0005] No protection against the above mentioned or other epicutaneously employed antigens was reported to date. Antibodies against diptheria or tetanus toxoid, and bovine serum albumin, which were generated by applying the antigens on the skin of BALB/c mice in combination with cholera toxin (Glenn et al., 1998, *op. cit.*) resulted in a very weak immune response without the adjuvant. Even after the inclusion of Cholera toxin (CT), the average specific antibody titre for diptheria and tetanus antigens was around 50x and between 70x and 4000x (depending on the inclusion of

individual data points), respectively, below that elicited by cholera toxin per se (Glenn et al., 1998, *op. cit*). The corresponding absolute respective titre values were 14±17 and 8±16; the anti-BSA titre was approximately 11±11 (average value +/- standard deviation as calculated from the published figures). No therapeutic or prophylactic effect was demonstrated for these low titres, which shows that the path towards simple non-invasive vaccination is not at all straightforward. The more recent paper published by the same group (Glenn et al., 1998b) demonstrated protection against CT after transnasal challenge which does not allow any conclusion with regard to protection obtainable by transdermal vaccination.

[0006] Earlier publications report on the deliverance of proteins across the skin, several orders of magnitude more efficient than in the above mentioned study, as judged by the titres, exploiting mechanosensitive and hydrosensitive, self-regulating carriers (Transfersomes) (for a review, see Cevc, 1997, *op. cit.*). For potent antigens this induced antibody titres that were comparable with those elicited by subcutaneous protein injections: in the case of BSA, the absolute titre of IgG was around 200 in either case (Paul, A., Cevc, G. Non-invasive administration of protein antigens. Epicutaneous immunisation with the bovine serum albumin. Vaccine Res. (1995) 4: 145-164) and for gap junction protein titres between 15.000 and 100.000 were measured (Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunisation with large proteins by means of ultradeformable drug carriers. Eur. J. Immunol. (1995) 25: 3521-3524; Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, Transfersomes. Vaccine (1997) 16: 188-195.). The review "Transferosomes, liposomes and other lipid suspensions on the skin" (Critical Rev. Therap. Drug Carrier 13, 257-388, 1996), inter alia, discusses DRV liposomes, as discribed in WO 92/04009, as immunoadjuvants in their own right. However, it is also disclosed that liposome-mediated penetration of cytokine IFN-gamma into the skin is inefficient and probably restricted to hair follicles. WO 91/01146 relates to immunogenic conjugates comprising a carbohydrate-containing antigen or other antigen bound to or genetically fused with a cytokine, lymphokine, hormone or growth factor. Yet, the generation of a protective immune response was not demonstrated in either of these publications and none of these publications provide for means and methods for the elucidation of a therapeutic or prophylactic immune response.

[0007] As is known today, the activity of Th1 or Th2 cells plays an important role in immune response: Th1 cells promote mainly the cell-mediated immunity, phagocyte-mediated host defence, but also the production of antigen specific IgG2a in mice. In contrast, Th2 cells tend preferentially to support phagocyte independent host-response, IgG1, IgE and IgA immunoglobulin generation.

[0008] The Th1 or Th2 basis of an immune response, that is, the differentiation into Th cell subtypes, not only depends on cytokines and the activity of other regulatory molecules (Luger, T. A., Schwarz, T. The role of cytokines and neuroendocrine hormones in cutaneous immunity and inflammation. Allergy (1995) 50: 292-302; Lohoff, M., Gessner, M., Bogdan, C., Roellinghoff, M. The Th1/Th2 paradigm and experimental murine *Leishmaniasis*. Int. Arch Allergy Immunol. (1998) 115: 191-202.); the nature of antigen presenting cells and antigen amount used also play an important role. Cytokines are produced transiently by almost all eukaryotic cells and act via specific cell-surface receptors. Indeed, every cell in the skin, after appropriate stimulation, can release such (glyco)protein factors or express their receptors. Most cytokines are pluripotent and can induce each other or else influence the expression of relevant receptors. This allows cytokines to act in synergistic, additive or antagonistic fashion, within the framework of so-called cytokine cascade (Luger & Schwarz, 1995; *op. cit.*).

[0009] The role of different cells in immunoactivation after cutaneous antigen application is as yet incompletely understood (Luger & Schwarz, 1995; *op. cit.;* Lohoff et al., 1998, *op. cit.*). Langerhans cells, located in the suprabasilar skin region, are believed to play the main role in immunopresentation. These cells first bind and process the antigens, then migrate from the epidermis into the lymphatic vessels, and further into the proximal, draining lymph node, bearing the digested antigens with them. During the process Langerhans cells undergo phenotypical and functional alterations and differentiate into (lymphoid) dendritic cells which finally offer the antigens to naive $CD4^+$ T cells that have entered the lymph nodes through the high endothelial venules. In contrast, the other two major types of antigen presenting cells in the skin, macrophages and B lymphocytes, first require activation in order to present antigens and stimulate T cells. Antibodies may be presented to T cells by the venular endothelial cells, and perhaps by certain basic cells of the skin as well.

[0010] It is clear, for example, that keratinocytes can augment the local inflammation by producing a plethora of proinflammatory cytokines, including IL-1α, GM-CSF and TNFα (Pastore, S., Fanales-Belaso, E., Abbanesi, C., Chinni, L.M., Giannetti, A., Girolomoni, G. Granulocyte macrophage colony stimulating factor is overproduced by keratinocytes in atopic dermatitis: Implications for sustained dendritic cell activation in the skin. J. Clin. Invest. (1997) 99: 3009-3017.). Keratinocyte derived cytokines are also critical for the maturation of Langerhans cells into potent antigen presenting cells (Nasir & Gaspari, 1996, *op. cit.*). The extent to which the former cells directly participate in antigen presentation (Kondo & Sauder, 1995, *op. cit.*) is unknown but the production of inhibitory cytokines, such as IL-10, non-functional IL-12 and TGFβ, by keratinocytes is an established fact (Nasir & Gaspari, 1996, *op. cit.*).

[0011] The fibroblast pool in the skin also contains cellular subsets that are involved in antigen processing. For example, one subset of fibroblasts is recruited selectively by cytokines at the inflammation site in scleroderma (Fries,

K.M., Blieden, T., Looney, R. J., Sempowski, G.D., Silvera, M.R., Willis, R. A., Phipps, R. P. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin. Immunol. Immunopathol. (1994) 72: 283-292.).

[0012] It has been reported previously that epicutaneous antigen application produces a different immune response than the more conventional routes of administration through the oral cavity or the nose. For example, after repeated epicutaneous ovalbumin exposure on the skin anti-ovalbumin IgE-s are prominent (Wang et al., 1996, *op. cit.*). Using bovine serum albumin as a model antigen on the skin, an unusually strong IgA production was previously observed (Paul et al., 1997, *op. cit.*), but no consistent picture of the interdependency between the details of epicutaneous antigen presentation and the resulting immune response emerged to date.

[0013] Numerous and different cells participate in mounting an immune response against the cutaneously delivered macromolecules. As has been stated above, the approaches taken so far have not led to the establishment of a convincing strategy for generating a protective immune response. This may be due to the fact that the prior art strategies, such as antigen injection, have not assisted in dissecting the immune response obtainable by applying antigens to the skin to an extent that allows for devising a directed and protective immune response. For example, it is known that antigen injection, as any lesion or other kind of skin perturbation, including the presence of chemical irritants, releases various cytokines from the skin (which not only is the heaviest organ in the body but also makes out the major part of the body immune system). This maximises the strength, but prevents the fine tuning, of cutaneous immune response, which is also sensitive to the nature of antigens used. High impact vaccine delivery profits from this effect.

[0014] Material transport across the skin by means of ultradeformable carriers is just the opposite of said high-impact delivery approach, as it reportedly does not affect the skin. It is believed that this is clue to the fact that such hydrosensitive, ultradeformable bodies - so called Transfersomes™ (Cevc, 1997, *op. cit.*), penetrate the stratum corneum through 'virtual channels' between corneocytes, adjusted to the shape of the cells (Schätzlein, A., Cevc, G. Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes). Br. J. Dermatol. (1998) 138: 583-592.). It was proposed that Transfersomes push the cells in the skin and intercellular lipids apart during the process, preferentially at the sites of weakest contact. The passages thus generated seem to be approximately 20-30 nm wide, on the average. They cover several percent (~4%) of the skin surface (Schätzlein & Cevc, 1998, *op. cit.*), the draining of adjacent surface not included. This is much more than the normal shunt area (~0.1%), which explains the quantitative differences between the anti-BSA titres measured after antigen administration with ultradeformable carriers (Paul & Cevc, 1995, *op. cit.*) or by using Cholera toxin as an adjuvant (Glenn et al., 1998a, b, *op. cit.*).

[0015] Virtual channels in the skin opened by the carriers appear to be sufficiently wide to let the carriers as well as material associated with them pass through the barrier without significantly perturbing the organ. However, repeated insulin delivery across the skin by means of ultradeformable carriers was found not to induce antibodies against the protein (Cevc, G., Gebauer, D., Schätzlein, A. Blume, G. Ultraflexible Vesicles, Transfersomes, Have an Extremely Low Permeation Resistance and Transport Therapeutic Amounts of Insulin Across the Intact Mammalian Skin. Biochim. Biophys. Acta (1998) 1368: 201-215.)

[0016] The technical problem underlying the present invention was therefore to establish a means that allows for the successful induction of a medically useful transdermal immune response. The solution to said technical problem is achieved by providing the embodiments characterised in the claims.

[0017] Accordingly, the present invention relates to a transdermal vaccine comprising (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a, preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity or exerts such an activity, either of which resulting in the desired, medically useful immune response, and (c) an antigen or an allergen.

[0018] As regards the above recited values of up to 99%, it is to be noted that values below 50 % of the former relative concentration are often used. Even more advantageously values below 40 rel-% or even around and below 30 rel-% are chosen, whereas with the droplets that cannot be solubilised by the more soluble component relative concentrations that exceed the above mentioned ones by the factor of up to 2 are preferred.

[0019] In the context of this invention, the term "pathogen" refers to an entity which through its presence in or on the

body leads to or promotes a pathological state which, in principle, is amenable to or could profit from a preventive, curative or adjuvant immunotherapy. This includes pathogens causing microbial diseases such as extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species; a number of bacteria and all viruses, which survive and replicate within host cells; this latter group encompasses mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including but not limited to hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox, (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and various fungi prospering inside host cells; parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites. The pathogens further include *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases. Pathogens in this invention, furthermore, are assumed to include, but are not limited to, the eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body which do not result from microbial infections. Parts of certain pathogens, especially various microbial toxins, have porin-like properties and, consequently, may have some capability to cross the mucosa or to increase the flexibility of penetrant membranes.

[0020] The term "specifically" in combination with "releases" or "induces" denotes the fact that the compound interacts with cells capable of releasing cytokines by a receptor-mediated triggering of this cytokine release or induction. This specific release or induction is in contrast to an unspecific release or induction that is, for example, obtained by an intradermal injection.

[0021] The term "allergen" is used in this invention to describe materials of endogenous or xenogenic, e.g. animal or plant, origin which result in an undesired immune response of the body exposed to such an allergen, often resulting in an acute hypersensitivity reaction. Allergising microbes or parts thereof (e.g. of mite), parts of plants (e.g. pollen) or animal (e.g. hair and skin debris), but also man made and inorganic substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the skin or not.

[0022] The term "(therapeutic) vaccination" in the context of this invention describes any kind of therapeutic immunisation, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

[0023] Immunisation denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

[0024] An "antibody" or an "immunoglobulin" denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their "derivatives" include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives. Fragments include e.g. single chain fragments, Fc-, Fab- $F(ab')_2$- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

[0025] An "antigen" is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system.

[0026] The term "cytokine", as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1α and IL-1β, tumour necrosis factor (TNF), transforming growth factor (TGF-β and -α), Type I and II interferons (IFN-α1, IFN-α2, (IFN-ω), IFN-β, IFN-γ), migration inhibitory factor, MIF, c-kit ligand, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

[0027] Cytokines that mediate natural immunity particularly well include type I interferons (IFN-α and IFN-β), tumour necrosis factor (TNF), interleukin-1 (IL-1 α and IL-1β), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. The process relies on antiproliferative (e.g. with IFN-s), proinflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, conse-

quently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic action.

[0028] Cytokines that mediate immune-mediated inflammation, which heavily relies on the cell-mediated response, are interferon-gamma (IFN-γ), lymphotoxin (TNF-β, interleukin-10 (IL-10), interleukin-5 (IL-5), interleukin-12 (IL-12) and, probably, migration inhibition factor. Leukocyte growth and differentiation are most affected by interleukin-3 (IL-3), c-kit ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage or granulocyte colony stimulating factor (M-CSF or G-CSF) and interleukin-7 (IL-7).

[0029] The term "immunoadjuvant" is used here to describe any substance which supports, augments, stimulates, activates, potentiates or modulates the desired immune response of either cellular or humoral type, specifically in the case of prophylactic treatment by increasing the antigen specific immune response of any kind and in the case of therapeutic treatment often by supporting cell-mediated immunity. This can be achieved by the addition of suitable cytokines, their blends or antagonists, or less directly by the chemical irritation of the skin, when this contributes directly or indirectly to the release of cytokines from the skin or other involved peripheral tissues, or else by catalysing or promoting the biosynthesis of the molecules in the tissue which then lead to such action, provided that the final outcome is an increased success of vaccination, that is of prophylactic and/or therapeutic action of used antigen. The class of immunoadjuvants which indirectly contribute to the useful cytokine pool includes small chemical entities with an allergenic potential, such as certain allergenic (metal) ions, including but not limited to LiCl, $HgCl_2$, molibdenum, acids, bases and other irritating compounds, such as dicyclohexylmethane-4,4'-diisocyanate, ditrocarb (diethyldithiocarbamate), 2,4-dinitrochlorobenzene, isoprinosine, isophorone-diisocyanate, levamisole, (phenyl)oxazolone and alike, Swansonine, sizofran, phthalic anhydride, thymopentin, (fatty) alcohols, (fatty) amines, (fatty) ethers, ricin, or other suitable amphiphiles, many surfactants and chemical skin permeation enhancers, as well as derivatives or combinations thereof; furthermore, (low molecular weight) fragments of or derivatives from microbes, including lipopolysaccharides (such as LPS), cord-factor (trehalose-dimycolate) and other polysaccharides attached to membranes, when used in sufficient quantity;, acetylmuramyl-alanyl-isoglutamin, and arger fragments of microbes, including bacterial exo- and endotoxins, or enterotoxins, such as cholera toxin and the heat labile toxin of *E. coli,* and their macromolecular fragments, such as A-chain derivatives, most, if not all, of which seem to posses ADP-ribosylating activity, the high potency immunoadjuvant LT holotoxin, etc., cell-wall skeleton, attenuated bacteria, such as BCG, etc. Less established examples include clostridial toxin, purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* outer membrane protein of group B *Neisseria meningitidis* (GBOMP), various other peptidoglycanes, etc. Immunoadjuvants, in other words, include molecules that alter the uptake or presentation of antigens, activate or increase the proliferation of antigen specific lymphocytes, or interfere with the dominant control mechanism in the immune response, not just in the skin but also in the other immunocompetent tissues. (The mucosal adjuvant activity of ADP-ribosylating bacterial enterotoxins is a well established and known example for this.) On the other hand, molecules which change the (relative) concentrations of cytokines or other immunoadjuvants, such as anti-immunoadjuvant antibodies or other agonists or antagonists of immunoadjuvants, also are immunoadjuvants in the sense of this invention. The same is true for molecules which affect lymphocyte homing, such as various selectins (LECAMS, e.g. various CD62-s), GlyCAM-1, MadCAM-1, VCAM-1, ICAM-1, hyaluronate, etc., and other chemokines, such as RANTES or MCP-1. Endogenous group of immunoadjuvant furthermore comprises histamines, transfer factor, tuftsin, etc.. As many of the above mentioned immunoadjuvants do not have sufficient potency to ensure the desired effect after the non-invasive immunisation at too low, and sometimes too high, concentration or on their own, the functional definition of an aduvant used in this work includes a fortiory sufficient and such modulation of cytokine concentration and distribution pattern in the body that results in mounting the desired therapeutic or prophylactic immune response. If required to gain clarity said modulation and its extent must be determined in a dedicated experiment, in which the specific cytokine levels are determined, for example.

[0030] "Immunoadjuvant manipulation" denotes a non-chemical treatment of the skin, such as skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound, field, etc., or even an injection of a non-immunogenic formulation in the skin, provided that such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

[0031] The term "immunogen" denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing an immune response.

[0032] "Immuno-tolerance" denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

[0033] Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

[0034] Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

[0035] The term "two forms of a substance" in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc..

[0036] "Non-invasive administration" or "non-invasive delivery" denotes application on or transport through an intact

barrier, in the biological applications dealt with in this disclosure, through intact skin.

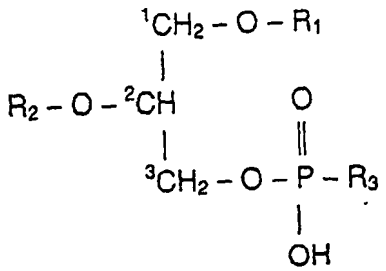
[0037] "Penetration" describes a non-diffusive motion of relatively large entities across a barrier. This process typically relies on the penetrant adaptation to the otherwise confining pores in the barrier and also may involve a penetration induced decrease in the barrier resistance, such as pore widening or channel opening; the process does not depend, however, primarily on the penetrant concentration gradient across the barrier.

"Permeation" refers to a diffusive motion across the semi-permeable barriers. The prime example for this is the transport of molecules or molecular aggregates under the influence of a permeating species concentration gradient across the barrier.

[0038] A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

[0039] For further definitions, especially such pertaining to the penetrants in terms of complex body deformability, the corresponding mechanism of action, lists of interesting penetrant ingredients or selected agents it is referred to the issued or pending patents (DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287). Detailed information relevant for the manufacturing process and penetrant loading with the antigenic (macro)molecules and/or immunoadjuvants, which are too big to permeate through the barrier, can be found in international patent application PCT/EP98/06750.

[0040] Typically, the less soluble amongst the aggregating substances forming a carrier is a lipid or lipid-like material, especially a polar lipid, whereas the substance which is more soluble in the suspending liquid and which increases the droplet adaptability belongs to surfactants or else has surfactant-like properties. The former ingredient, typically, is a lipid or lipid-like material from a biological source or a corresponding synthetic lipid or any of its modifications, such lipid often belonging to the class of pure phospholipids with the chemical formula



where R_1 and R_2 is an aliphatic chain, typically a C_{10-20}-acyl, or -alkyl or partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4}-alkyl, C_{1-5}-alkyl substituted with carboxy, C_{2-5}-alkyl substituted with hydroxy, C_{2-5}-alkyl substituted with carboxy and hydroxy, or C_{2-5} -alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, in particular half-protonated fluid fatty acids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebrosides, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with or else with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyllipids.

[0041] The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyldimethyl-aminoxida, esp. a dodecyl-

dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N- dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl, -myristoyl, -cetylstearyl, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate, -linoleate, -linolenate-, - palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkyl-sulphate (salt), e.g. in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g. lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecyl-phosphatidylcholine, or else is a surface-active polypeptide. It is important to realise, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionisation or salt states of polar lipids differ widely in their properties. It therefore stands to reason that two different physicochemical states of the same (polar) lipid mixed together in a membrane will produce a highly deformable carrier satisfying the conditions of this work.

[0042] More general information on lipid suspensions can be found in handbook dealing with 'Liposomes' (Gregoriadis, G., Hrsg., CRC Press, Boca Raton, Fl., Vols 1-3, 1987), in the book Liposomes as drug carriers' (Gregoriadis, G., Hrsg., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids which can be used conveniently to prepare biocompatible immunopenetrants are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

[0043] It may be convenient to adjust the pH value of a formulation immediately after preparation or just before its application. Such an adjustment should prevent the deterioration of individual system components and/or drug carriers under the conditions of initial pH without sacrificing physiological compatibility. To neutralise a penetrant suspensions it is reasonable to use biocompatible acids or bases to prepare buffers with a pH value between 3 and 12, frequently between 5 and 9 and most often between 6 and 8 Physiologically acceptable acids are, for example, diluted aqueous solutions of mineral acids, such as hydrochloric acid, sulphuric acid, or phosphoric acid, or organic acids, such as carboxyalkane acids, e.g. acetic acid. Physiologically acceptable bases are, for example, diluted sodium hydroxide, suitably ionised phosphoric acid, etc.

[0044] If required, immunogen suspension can be diluted or concentrated (e.g. by ultracentrifugation or ultra-filtration) before the application; additives can also be given into the suspension at this time or before. The additives are often selected amongst substances that reduce the formulation sensitivity to ambient stress, including microbicides, antioxidants, antagonists of undesired enzyme action, in case cryo-preservants, thickening agents, etc.. However, after any system manipulation, the carrier characteristics should be checked and, if required, readjusted.

[0045] In accordance with the present invention it was surprisingly found that macromolecular antigens associated with ultradeformable lipid aggregates described herein (immuno-penetrants) can cross artificial porous barriers as well as the skin, despite the fact that the average diameter of said penetrants exceeds the average pore/channel diameter, and that such immuno-penetrants can elicit a therapeutic or prophylactic immune response, provided that said immuno-penetrants are associated with compounds that display cytokine activity or induce the generation in and/or the release of cytokines from the skin, and/or other immunocompetent organs in the body. Alternatively, said compounds antagonize cytokine activity. This latter embodiment advantageously directs the immune response into a Th1 or Th2 dependent immune response by blocking the respective other route. The antigen carriers described in this invention maintain sufficient stability before and during the process. It was further surprisingly found that the resulting immune response is not directly proportional to the applied dose, which implies that the amount of antigen may be varied. Said amount should be well chosen for optimum effect. Choosing optimum amount or range of antigen is well within the skills of the person knowledgeable in the art taking into account the teachings of this specification.

[0046] The at least bi-component immuno-aggregates used as carriers in the vaccines of this invention excel in high deformability and most often have the form of vesicles a with highly flexible membrane. Although such carriers have been employed in immunisation protocols before, it was unexpected that the previously postulated carrier immunoadjuvancy does not eliminate the need to include compounds with cytokine activity or the appropriate antagonists thereto, preferably of IL-4, IL-10, TGF-β, IL-5, IL-6, IL-9 and IL-13; or, after the pre-stimulation of T-cell receptor, also of IL-1 in order, to achieve the desired protective immune response. By the same argument, IL-12, IFN-γ and lymphotoxin (LT or TNF-β) are advantageously included to promote the Th1 response and thus to favour the cell-mediated immune

response and to provide a means for treating viral and other parasite diseases or for promoting the immuno-tolerance. For example, a combination of IFN_γ, IL-12 and anti IL-4 is expected to revert Th2 response toward Th1 type. More broadly speaking, increasing the relative amount of IL-12 and IL-4 in the beginning of an immune response in favour of the former is proposed to be useful to promote Th1 response also in the case of penetrant mediated immunisation, and *vice versa*, whereas IL-2 is going to support NK and B cells growth, to stimulate antibody synthesis, and to affect the magnitude of T-cell dependent immune response in general. Thus, whereas the prior art demonstrated that antibody titres could be induced by using suitable carriers in combination with antigen and optionally with immunoaduvants, the immuno responses obtained were not demonstrated to be protective.

[0047] A particular advantage of the present invention is due to the fact that it was surprisingly found that the transfer of the penetrant described in this invention does not lead to an essential disturbance of the cytokine composition within the skin. In other words, the transfer of these carriers through the skin will per se not induce any essential release of cytokines. It is therefore possible to study now and to trigger a desired immuno response by including into the vaccine of the invention a compound that specifically induces or releases cytokines from cells in the skin or other organs that are competent to release such cytokines. Fine tuning of a desired immuno response thus may be possible. Alternatively, a compound having or exerting cytokine activity can be included into the vaccine of the invention. Further, an antagonist of cytokine activity may be used that specifically prevents the action of such cytokines. In this embodiment, the immuno response may advantageously be directed towards the Th1 or Th2 pathway. It is important to note that these compounds specifically induce or release cytokines in dependence on antigen properties. They are thus distinguished from adjuvants which, in accordance with the present invention, unspecifically and broadly support an immuno response.

[0048] Application of the vaccine of the present invention allows, in conclusion, therefore the fine tuning of a desired immuno response to a given antigen, the nature of which also plays a given role, of course. This immuno response may be enhanced by an unspecific immuno response, triggered, for example, by an adjuvant. The option of fine tuning the immuno response is in particular advantageous over the prior art using only the injection of vaccines because the injection process per se will heavily and unspecifically disturb the relative cytokine concentrations in the skin.

[0049] Consideration of the above mentioned criteria not only provides the basis for a suitable kind of immunopresentation to the cells of the skin and peripheral immune system (by the carriers) but also ensures such immunoprocessing that will either predominantly generate antigen-neutralising antibodies in the body, will give rise to the cell-mediated immune response, or else will result in the gradual development of tolerance against the antigens or in the specific promotion of cell-mediated immunity.

[0050] In accordance with the present invention, it was also found that the outcome of noninvasive transcutaneous vaccination is strongly affected by the immunopenetrant (antigen carrier) composition. Using antigens of different purity, unexpectedly, resulted in vastly different immune response. This was reflected in the observation that organisms with a similar overall titre revealed diverse levels of protection, probably due to the different final antigen isotype patterns.

[0051] Furthermore, the addition of a conventional, low molecular weight immunoadjuvant, monophosphoryl lipid A, not only made the result of epicutaneous immunisation more robust, as documented by the smaller standard deviation in the measured antibody titres published before. Using this immunoadjuvant in immuno-carriers also, unexpectedly and contrary to previous experience obtained in mice, increased the secretion of IgG2b, and less strongly of IgG2a, but did not enhance IgA production. As the presence of IgG1, which is a Th1-like immunoglobulin, is inferred to be essential for, at least murine, protection against the tetanus toxin, the role of lipid A or bacterial antigens was thus revealed for the first time. For the future medical and commercial use of teachings disclosed in this invention it is important to realise that a high (specific) antibody titre does not necessarily imply a good protection result; to achieve the desired and sufficient protection the right kind and relative amount of certain antibody isotypes is required, such that will give prevailantly Th1- or Th2-type of immune response (see previous discussion), as the case should be.

[0052] Basic formulations suitable for achieving the desired goals are known in the art; see, e.g., DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287, for more detailed or complementary information. The vaccine of this invention is not useful just for prophylactic or therapeutic vaccination but, moreover, is applicable for the treatment of allergy and for obtaining immunity against microbes, including extracellular and intercellular bacteria, viruses and parasites in the human and veterinary medicine.

[0053] In combination with the above mentioned penetrants, an antigen, such as an immunoactive substance, is transported across the barrier in form of a physical or a chemical complex with the former.

[0054] In order to profit from the pool of cytokines residing in the skin, a particularly useful method of vaccination is proposed in which an immunogen is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation as defined before.

[0055] It is particularly advantageous to use the readings from the above mentioned local immune response to a patch assessment for optimising the details and the course of further allergen administration, and thus to positively affect the outcome of therapeutic or prophylactic vaccination. It is believed that such an approach could be used advantageously to reach or improve immuno-tolerance of the tested subject to an applied allergen.

[0056] If primary immunisation is done invasively, typically by using a subcutaneous injection or some other suitable

skin barrier perforating/destructing method, , one expects to obtain high IgM levels but the subsequent, booster immunisations may then be done non-invasively as described in this invention.

[0057] Finally, several optimisation methods are proposed which can be used to improve immunogens and vaccination based on highly deformable penetrants. Preferred is a method wherein the flux of penetrants associated with an immunogen through the various pores in a well-defined barrier is determined as a function of suitable driving force or pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further. Its core is the determination of the flux of immunopenetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which acts across the barrier, and the resulting data analysis in terms of a characteristic curve which, in turn, can be employed to optimise the formulation or application further, based on comparison of different data sets. This includes comparison with the results pertaining to the immunogen-free penetrant suspensions of known skin penetration capability, reported for example by Cevc et al., (1998, *op. cit.*). In a complementary, preferred, embodiment various combinations of immunomodulants or of immunomodulating procedures are tested with regard to chiefly Th1- or Th2-related cytokine production and the results are then used to make a suitable choice for the final therapeutic or prophylactic application.

[0058] Vaccination is typically done at ambient temperature, but lower or higher temperatures may also be suitable. They make particular sense with the formulations comprising synthetic substances which are rigid between the room and the skin or other barrier temperature.

[0059] Manufacturing temperature is normally chosen in the 0 to 95 °C range. Preferably, one works in the temperature range 10-70 °C, most frequently at temperatures between 15 °C and 45 °C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in composition or physical state. The skin temperature is normally 32 °C. Other temperature ranges are possible, however, most notably for the systems containing freezable or non-volatile components, cryo-or heat-stabilised formulations, etc.

[0060] If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in cold (e.g. at 4°C), with or without an associated antigen. Manufacturing and storage under an inert atmosphere, e.g. under nitrogen, is possible and sometimes sensible. The shelf-life of immunogen formulation can also be extended by using substances with only a small number of double bonds, that is, by a low degree of unsaturation, by the addition of antioxidants, chelators, and other stabilising agents, or by preparing the immunopenetrants ad hoc or *in situ* from a freeze dried or a dry mixture.

[0061] In a preferred embodiment of the vaccine according to the invention the compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity and the antigen are associated with the penetrant.

[0062] In a further preferred embodiment of the vaccine according to the present invention the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

[0063] In an additional preferred embodiment of the vaccine according to the present invention the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.

[0064] The invention in one further preferred embodiment relates to a vaccine wherein total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 w-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.

[0065] In another preferred embodiment of the vaccine according to the present invention total antigen concentration is between 0.001 w-% and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.

[0066] In another preferred embodiment of the vaccine according to the present invention the formulation further comprises (da) a low molecular weight chemical irritant, and/or (db) a low molecular weight compound from a pathogen or a fragment or a derivative thereof.

[0067] In yet another preferred embodiment of the vaccine according to the present invention the compound exerting cytokine activity is IL-4, IL-2, TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, preferably IFN-α or IFN-β, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

[0068] In one more preferred embodiment of the vaccine according to the present invention the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.

[0069] The term "active fragment or derivative thereof" in this connection means that the above-recited activity is essentially maintained or mimicked by the substance used.

[0070] In another preferred embodiment of the vaccine according to the present invention the antigen is derived from a pathogen.

[0071] In another particularly preferred embodiment of the vaccine according to the present invention said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-

negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.

[0072] In a preferred embodiment of the vaccine according to the present invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant which leads to an acute hypersensitivity reaction of the body exposed to the allergen, many such allergens stemming from mite, polen, animal hair or skin debris, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

[0073] In a further preferred embodiment of the vaccine according to the present invention the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen and immunoadjuvant chosen, performed by subcutaneously injecting the formulation or performing the tests *in vitro,* and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

[0074] In a different preferred embodiment of the vaccine according to the present invention the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo-and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli,* an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

[0075] In a particularly preferred embodiment of the present invention said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.

[0076] In another particularly preferred embodiment of the vaccine according to the present invention the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

[0077] In still another particularly preferred embodiment of the vaccine according to the present invention the low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines, (fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

[0078] In a preferred embodiment of the vaccine according to present invention the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or comparable subject is deemed to be unacceptable owing to the local irritancy, as assessed by the methods and standards commonly used to test such an irritant.

[0079] In a further particularly preferred embodiment of the vaccine according to the present invention the allergen belongs to the class of inhalation allergens, including various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; food and drug allergens; contact allergens; injection, invasion and depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., parts of implantation material, etc..

[0080] In a preferred embodiment of the vaccine according to the present invention the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunisation, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.

[0081] In another preferred embodiment of the vaccine according to the present invention the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2}, even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2}, and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2}. It may also be advantageous to use different administration areas to control the applied immunogen dose, using easily accessible or sheltered body areas (such as the chest or back regions, arms,

lateral side of the neck, e.g. behind the ears, or even in the scalp region) for the purpose.

[0082] In a different preferred embodiment of the vaccine according to the present invention said antigen is a pure or purified antigen. The use of highly purified antigens in the vaccine of the invention has turned out to be particularly advantageous for the generation of a protective imrnuno response.

[0083] The present invention further relates to a kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine.

[0084] In a preferred embodiment according to the present invention the kit comprises at least one injectable dose of the antigen described above.

[0085] The present invention further relates to a method for generating a protective immune response on a mammal comprising vaccinating said mammal with a vaccine as described above.

[0086] In another preferred embodiment of the method according to the present invention different treatment areas are selected to control the applied immunogen dose and the outcome of therapeutic vaccination.

[0087] In one more preferred embodiment of the method according to the present invention a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.

[0088] In a different preferred embodiment of the method according to the present invention the vaccine of the present invention is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

[0089] In a preferred embodiment of the method according to the present invention immunogen is applied in a non-occlusive patch. This embodiment can also be used for the purpose of assessing the skin reaction to an epicutaneously administered immunogen in the penetrant suspension, to which the former, at least originally, is allergic and which thus gives rise to an acute local hypersensitivity reaction, as seen, for example from the resulting flare, irritation, etc.

[0090] In another preferred embodiment of the method according to the present invention at least one dose of vaccine is administered.

[0091] This embodiment of the method of the invention includes the repeated administration of the vaccine of the invention. Repeated administration includes repeated administration on the skin or one or more administrations on the skin in combination with e.g. parenteral administrations. In this connection, the kit of the invention may be advantageously used that comprises one or more containers or ampules comprising the vaccine of the invention.

[0092] In a particularly preferred embodiment of the method according to the present invention said vaccine is administered as a booster vaccination.

[0093] In a most preferred embodiment of the method according to the present invention the primary immunisation is done invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destructing method, and the at least one subsequent, booster immunisation is done non-invasively.

[0094] In a preferred embodiment of the method according to the present invention the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.

[0095] In a particularly preferred embodiment of the method according to the present invention the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years. In a further preferred embodiment, repeated immunogen administration is advocated to maximise the final effect of a therapeutic vaccination. It is proposed to use between 2 and 10, often between 2 and 7, more typically up to 5 and most preferred up to 3 immunisations, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined as described above or another suitable assessment method, or else to deem the effort as having failed. The time interval between subsequent vaccinations should preferably be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years, when a subject is being immunised for the first time. Rodents, such as mice and rabbits are advantageously immunised in 2 weeks interval, primates, e.g. monkeys and often humans, need a booster vaccination in 3-6 months interval.

[0096] In a preferred embodiment of the method according to the present invention the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

[0097] The invention finally relates to the use of the transdermal carrier, the compound which specifically releases

or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined hereinbefore for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

[0098] The disclosure contents of the documents cited throughout this specification are herewith incorporated by reference. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of Innovative Dermale Applikationen GmbH and bearing the title "Transnasal transport/immunisation with highly adaptable carriers".

[0099] The figures show:

[0100] Figure 1 gives the data on survival of animals immunised epicutaneously with mixed micelles or Transfersomes loaded with TT, to illustrate aggregate size (stability) effect, since the over-destabilised Transfersomes normally disintegrate into the mixed lipid micelles.

[0101] In figure 2 the comparison is made between the immune response to conventional lipid vesicles (liposomes) and ultradeformable lipid vesicles (Transfersomes) carrying TT and applied on the skin, the information on corresponding specific antibody concentrations in serum (expressed as absorbance) being given in upper panel.

[0102] Figure 3 illustrates the effect of increasing antigen dose on the outcome of epicutaneous immunisation by means of Transfersomes, the results being expressed as absorbance change, antibody titre, or animal survival, together with the corresponding specific antibody isotyping data.

[0103] Figure 4 highlights the effect of antigen purity on the result of epicutaneous immunisation with tetanus toxoid in Transfersomes, including information on time dependence of animal survival.

[0104] Figure 5 compares the outcome of repeated invasive (subcutaneous) and non-invasive (epicutaneous) immunisation by means of TT in Transfersomes, including animal survival, serum concentration (in terms of absorbance), specific antibody titre, and antibody distribution pattern values.

[0105] Figure 6 illustrates the effect of skin pre-treatment (non-specific challenge) on the immune response following Transfersome mediated TT delivery across the skin.

[0106] Figure 7 focuses on adjuvant effect of a relatively low-molecular weight immuno-stimulator, monophosphoryl Lipid A (LA), delivered across intact skin together with TT in Transfersomes.

[0107] Figure 8 demonstrates the immuno-adjuvancy of a cytokine, interleukin-12 (IL-12) transported across the skin together with TT by means of Transfersomes.

[0108] Figure 9 deals with the immuno-modulation by various cytokines of the murine response against TT antigen delivered in Transfersomes non-invasively through the skin.

[0109] Figure 10 presents experimental evidence for the immune response stimulation of mice treated on the skin by TT in Transfersomes, when the carriers also include cholera toxin (CT) to support the specific antibody production, and thus animal protection against an otherwise lethal challenge by the tetanus toxin.

[0110] The documents cited in this specification are incorporated herein by reference.

[0111] The examples illustrate but do not define the limits of the invention.

General experimental set-up and sample preparation

[0112] ***Mice*** *of Swiss albino* strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunisation and were normally kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. One day prior to an immunisation, the application area on murine back was shaved carefully. The antigen was administered with a high precision pipette on the skin surface and left to dry out partially. To prevent immunogen abrasion, the animals were transferred into individual cages in which they were kept for 18 hours following each epicutaneous material administration.

[0113] General anaesthesia was used to keep the test animals stress free and quiet during manipulations, including immunisation. An injection of a mixture of Ketavet and Rompun (0.3 mL per mouse of an isotonic NaCl solution containing 0.0071 % Rompun (Bayer, Leverkusen, Germany) and 14.3 mg/mL Ketavet (Parke-Davis, Rochester, N.Y) into the peritoneal cavity was used for the purpose. This typically kept the animals asleep for app. 2 hours.

Immunogens.

[0114] Ultradeformable immuno-carriers, or immuno-penetrants (immuno-Transfersomes), studied in this work, typically had the form of (oligo)bilayer vesicles. They contained biocompatible (phospho)lipids, such as phosphatidylcholine, and (bio)surfactants, such as sodium cholate or polysorbate (Tween 80), different compositions maintaining the high aggregate deformability being possible. Additional ingredients were monophosphoryl lipid A, with a versatile immunoadjuvant activity, and antigens, as required and specified.

[0115] *Conventional vesicles, liposomes,* comprised soy phosphatidylcholine (SPC; Nattermann Phospholipids,

Rhone-Poulenc Rorer, Cologne, Germany) and were prepared as described as follows. An organic lipid solution with or without the adjuvant monophosphoryl lipid A (MLA) at 0.04 mol-% relative to SPC was first dried under vacuum (10 Pa, overnight). The resulting lipid film was hydrated with a solution of tetanus toxoid (2.0 mg/mL; Accurate antibodies, NY, USA) in phosphate buffer (pH = 6.5) to get a 10 wt-% lipid suspension. Crude suspension of lipid vesicles was extruded through the series of polycarbonate membranes with 800 nm, 400 nm, and 200 nm pores, to narrow down the final vesicle size distribution.

[0116] *Highly deformable vesicles, Transfersomes,* were prepared as described earlier (Paul et al., 1995 *op. cit.*). In short, an ethanolic SPC solution was mixed with sodium cholate (Merck, Darmstadt, Germany) (3.75/1 mol/mol) and the adjuvant, if required. The mixture was dispersed in 10 mM phosphate buffer (pH = 6.5). This was done with tetanus toxoid present in the solution to give between 0.25 mg and 2.0 mg protein per 1 mL of suspension, as required. Vesicle suspension was then frozen and thawed three times. Subsequently, the formulation was passed through a microporous filter (200 nm; Poretics, CA) under pressure. To check the reproducibility of vesicle manufacturing, the optical density at 400 nm was measured with each preparation and confirmed to be approximately constant.

[0117] By varying surfactant-to-lipid ratio the vesicular aggregate deformability was controlled, up to the concentration at which membranes became unstable, owing to the high surfactant concentration, and reverted into a micellar form. Lipid vesicles without the surfactant added, which are commonly known as liposomes and have at least 10x less flexible membranes than Transfersomes, were used as negative controls.

[0118] Total lipid concentration was typically 10 w-%, unless stated otherwise. Antigen concentration was typically, but not necessarily, of the order of 1 mg/mL. A buffer containing microbicide provided the bulk phase. For other suitable compositions the expert is explicitly referred to other publications and patents from our laboratory.

[0119] ***Immunisations*** were done with different formulations, including the ultradeformable vesicles without antigens; such vesicles then contained the tetanus toxoid (with or without lipid A) and free immunogen. Each formulation was tested on six mice, unless stated otherwise.

[0120] In the case of subcutaneous immunisation, 40 μg of immunogen was injected per mouse. For a non-invasive administration, tetanus toxoid doses between 1 μg and 80 μg, associated with different carriers, were administered per mouse on the intact skin of upper dorsum. All non-injected formulations were applied with a high precision pipette and left to dry; during this period mice were kept in separate cages to minimise the applied material abrasion, such as might result from the rubbing of the murine backs on each other. Animals were boosted every two weeks, that is on days 14 and 28; the total immunisation scheme thus consisted of three doses, and comprised a prime and two boosts.

[0121] Animals were bled retro-orbitally on the days 7, 21 and 35. The collected blood was first allowed to clot. After a brief centrifugation in a micro-centrifuge the serum was separated, de-complemented at 56 °C for 30 min, and then stored at -20 °C, until the total antibody concentration and the specific antibody isotypes was determined.

[0122] ***Absorbency measurements*** were done using standard UV-vis spectrometer.

[0123] ***Measurement of tetanus toxoid (TT) specific antibodies in serum by ELISA.*** The level of anti-tetanus antibodies was determined by ELISA in the customary fashion, typically in a duplicate. In brief, ELISA plates (maxisorp: NUNC, Germany) were coated with an aliquot (100 μL containing 10 μg of TT/mL) in coating buffer (Na_2CO_3/$NaHCO_3$, pH=9.6) for 3 hours at 37 °C. Wells were first washed thrice with 200 μL/well of washing buffer and then blocked with 2% milk in washing fluid (for 1000 mL, 8 g NaCl, 1.45 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl and 0.05 % Tween-20) for 3 hours at 37°C. After single wash with 200 mUwell of washing buffer, the plates were incubated with various dilutions (1/50 to 1/6400) of the test serum. After an overnight incubation at 4 °C the plates were washed thrice with 200 μL/well of washing buffer and incubated with 100 μL of secondary antibody. When determining the amounts of IgG, IgA, or IgM, horse radish peroxidase (hrp) conjugated to the appropriate Anti-Ig was used. After a 3 hours incubation at 37 °C, the plates were washed thrice with 200 μL/well of washing buffer and the color was developed using o-phenyl diamine as hrp substrate. 0.4 mg/mL of o-phenyl-diamine in phosphate-citrate buffer (pH 4.5) with 0.4 pL H_2O_2 per mL was used for the purpose. After 2 minutes the reaction was stopped by the addition of 50 μL of 2N H_2SO_4. The absorbency was measured at 492 nm.

[0124] The method used to detect various isotypes was also ELISA based. It relied on the peroxidase-labeled, affinity purified secondary antibodies specific for IgG1 (1:1000), IgG2a (1:1000), IgG2b (1:1000), and IgG3 (1:200) which were all obtained from ICN ImmunoBiologicals. Further secondary antibodies included IgA (1:1000) and IgM (1:1000) linked to horse-radish peroxidase (Sigma, Neu-Ulm, Germany). The correspondingly labelled anti-mouse IgE was purchased from PharMingen (San Diego, CA. The antigens were again permitted to adsorb on test plates and incubated with the test serum after excess of the antigen had been washed away. Subsequently, 100 μL of appropriate specific secondary antibody solution was added to one of the six different plates, to determine anti-IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, respectively. The plates were incubated for 3 hours at 37 °C and processed further as described in previous paragraphs.

[0125] ***Challenge with antigen (the tetanus toxin) in vivo.*** On the day 35, test animals were challenged by injecting 50 times the LD_{50} of the tetanus toxin subcutaneously (s.c.). (The actual value of LD_{50} was fixed in separate experiments, during which a group of 16 weight-matched animals was challenged s.c. with increasing amounts of toxin and the number of survivors was determined.) To determine the acute TT toxicity in vaccinated animals, the clinical status

of such test mice was recorded for 4 days after the first challenge.

[0126] Non-protected mice showed signs of paralysis after 24 hours resulting in death, after 36 hours, at latest. Animals which developed no symptoms of paralysis or other anomaly over a 4 days period following the challenge were deemed immune against tetanus.

[0127] The long-term immunity was tested by challenging all immunised mice on a monthly basis with a dose of toxin corresponding to 50 times LD_{50}, for at least half a year.

Examples 1-2:

Aggregate size (stability) effect

[0128] Highly deformable vesicles (Transfersomes™: IDEA):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA, LA) relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0129] (Mixed lipid) Micelles:

65 mg phosphatidylcholine from soy bean (SPC)
35 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0130] Tetanus toxoid (2 mg/mL; Accurate Antibodies) used at the dose of
40 μg (20 μL) or 80 μg (40 μL) TT per mouse and immunisation

[0131] Application area: 1 cm^2 or 2 cm^2 for 40 μg or 80 μg TT per mouse on the upper dorsum.

[0132] To test the effect of formulation stability on the immunological properties of various, epicutaneously administered formulations, two kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) and relatively small micelles (diameter below 50 nm). The latter were chosen in the expectation that under sub-optimal conditions (owing to the lipid degradation or inappropriate aggregate composition) the latter may arise from the former.

[0133] **Antibody titres,** as reflected in the serum absorbency at 492 nm, are shown in figure 1. They show that mixed lipid micelles are less efficient antigen carriers than ultradeformable mixed lipid vesicles (Tfs) loaded with the same amount of TT. Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) were even less efficient immune response mediators.

[0134] **Animal protection data** reveal similar trend, as is seen in lower panel of figure 1.

Examples 3-4:

Aggregate deformability effect

[0135] Conventional lipid vesicles (liposomes):

100 mg phosphatidylcholine from soy bean (SPC)
0.4 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.5mL phosphate buffer, 10 mM, pH 6.5
2 mg/mL tetanus toxoid (Accurate Antibodies)

[0136] Highly deformable vesicles (Transfersomes™):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5

0.1 mL ethanol

[0137] Tetanus toxoid used at the dose of 40 µg or 80 µg TT/ mouse/ immunisation

[0138] Application area: 1 cm^2 or 2 cm^2 for 40 µg or 80 µg TT/ mouse/ immunisation on the upper dorsum.

[0139] **Results** obtained with the conventional vesicles differ from the data measured with highly deformable vesicles: simple liposomes, which do not cross the narrow pores in a barrier also do not elicit a substantial antibody titre. Conversely, the vesicles with a highly flexible and deformable, and thus better adaptable, membrane which were shown separately to move through the narrow pores in a barrier with greater ease, generate an appreciable quantity of antibody when applied on intact skin, according to the results of serum absorbency measurements (cf. figure 2).

Examples 5-10:

Antigen dose effect

[0140] Highly deformable vesicles:

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol
Tetanus toxoid (TT: Accurate Antibodies, New York, USA) concentration:
empty, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, giving raise to 0 µg, 10 µg, 20 µg, 40 µg or 80 µg TT/ mouse/ immunisation

[0141] Application area: 1 cm^2 for 0 µg, 10 µg, 20 µg, 40 µg and 2 cm^2 for 80 µg TT/mouse/ immunisation on the upper dorsum.

[0142] The results of this experimental series are illustrated in figure 3. It clearly shows the increase in immune response to epicutaneously administered tetanus toxoid in ultradeformable carriers with increasing TT dose. This is reflected in serum absorbency (up to the dose of 20 µg/immunisation), in specific antibody titre (up to the dose of 40 µg/immunisation), and in the survival data (which do not saturate for doses up to 80 µg/immunisation).

[0143] Less clarity is found in isotype distribution pattern, except for IgG1 (with a strong indication for the response saturation) and for IgG2b (perhaps, with the saturation between 40 µg and 80 µg per immunisation). IgM shows dose dependence similar to that of IgG1. The picture obtained for IgG2a is confusing.

Examples 11-13:

Antigen purity effect

[0144] Highly deformable vesicles:

as described with examples 5-10 (except in that the group treated with impure TT did not receive immunoadjuvant lipid A)

[0145] Tetanus toxoid: 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation

[0146] Application area: 2 cm^2 on the upper dorsum.

[0147] Antigen purity strongly affects the level of murire protection against tetanus toxin when the toxoid has been applied non-invasively on the skin. (Similar results obtained with injected antigen are not shown).

[0148] To substantiate the above mentioned statement" the medium filtrate from a culture of *Clostridium tetani* grown in vitro first was used as an impure antigen. To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times. Purified toxoid was purchased from Accurate Antibodies, NY, USA.

[0149] Swiss albino mice (n = 6) were immunised with identical nominal dose of impure antigen, with partially purified antigen supplemented with monophosphoryl lipid A or with purified antigen with monophosphoryl lipid A added. The antigen was always associated with similar Transfersomes. The composition and the method of preparation for the latter were the same are as described with previous examples. The details of immunisation schedule, bleeding times and challenge as well as analysis details were also similar to those mentioned before.

[0150] The results are given in figure 4. They demonstrate the role antigen purity plays in determining the quality as

well as the strength of an immune response against TT. The data shown in figure 4, moreover, indicate that the absorbency of even the specific antibody titre is not a reliable predictor of the therapeutic, that is, of prophylactic effect of an epicutaneous vaccination. This is due to the big differences in specific antibody isotypes which only contain a substantial proportion of Th1-like IgG2b compared to Th2-like IgG1 component if sufficiently pure antigen is used (see also page 12).

Examples 14-15 :

Comparison of epicutaneous and subcutaneous administration

[0151] Highly deformable vesicles, Transfersomes™ (IDEA):
as described with examples 5-10

[0152] Tetanus toxoid dose:

80 µg TT per epicutaneous immunisation
(using 2 mg TT/mL and application area of 2 cm^2)
40 µg TT per subcutaneous injection (using 2 mg TT/mL)

[0153] Using the same experimental procedures as described with examples 1-4, as appropriate, the antibody-specific serum titre, the level of animal protection against tetanus toxin and relative occurrence of different specific antibody isotypes was determined.

[0154] The results are given in figure 5. While the immunisation dependent increase in serum absorbency is comparable after invasive and non-invasive antigen administration the titre in the latter case is somewhat lower by the factor of 6 after primary immunisation and by the factor of 8 after second boost. Likewise, while the TT-specific levels of Th2-indicating IgG1 are similar in both arms of this experiment, the specific readings for other antibody subtypes, especially for IgG2a and at early time points also for IgG2b are by the factor 25 and 3, respectively, higher after antigen injections. However, the likelihood for the test mice to survive a subsequent challenge with a normally lethal dose of injected tetanus toxin is independent of the route of antigen administration, within the framework of this experimental series at least.

Examples 16-17:

Adjuvant skin treatment (pre-injection) effect

[0155] Highly deformable vesicles, Transfersomes™ (IDEA):

89.3 mg phosphatidylcholine from soy bean (SPC)
10.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0156] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation of 6 Swiss albino mice per group using impure antigen

[0157] Application area: 2 cm^2 on the upper dorsum.

[0158] Transcutaneous transport of macromolecules associated with Transfersomes across the skin seems to be extremely gentle; it therefore fails to trigger the immune system toward Th2-like immune response, if the antigen is used in a low amount or is impure. To change the situation, the skin can be (pre)stimulated to release corresponding messenger molecules from the organ prior to the actual non-invasive antigen administration by means of ultradeformable vesicles. For this purpose we have pre-injected the application site with 0.1 mL of saline, or a mild formulation of non-antigenic vesicles, prepared from biodegradable material of similar composition as the antigen carrying vesicles one day before using the latter. For additional control, incomplete Freund's adjuvant was also injected in different animals 24 hours before the application of immuno-carriers on the skin.

[0159] Illustrative examples of results are given in figure 6. They reveal higher specific antibody titres, especially for and improved protection in the mice that were pre-treated by injections rather than carrier formulation on the skin, which served as a control. The effect of incomplete Freund's adjuvant is surprisingly weak.

[0160] It is noteworthy that the serum absorbency or the specific antibody titre and animal survival, that is protective vaccination effect, are not correlated.

Examples 18-21:

Low molecular weight adjuvant (lipid A) effect

[0161] Highly deformable immuno-modulated TT-Transfersomes™ (IDEA):

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (LA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0162] Highly deformable standard immuno-vesicles, TT-Transfersomes™ (IDEA):
as above but without LA added

[0163] Tetanus toxoid: 2 mg/mL, with 20 µL or 40 µL corresponding to 40 µg or 80 µg TT per immunisation

[0164] Application area: 1 cm^2 or 2 cm^2, respectively, on the upper dorsum.

[0165] We believe that immuno-active, typically immunopotentiating, molecules must be present in the skin at the time of antigen presentation to the body by TT carriers that have crossed the barrier in order to achieve the desired immunological action of the antigen. To substantiate this conclusion we compared the outcome of non-invasive immunopresentation of TT by means of Transfersomes with or without a well known immunostimulant, monophosphoryl lipid A (LA), which is known to elicit generation of TNF in the body, for example. Two different antigen doses were used. In either case substantial titres and a measurable prophylactic immune response (partial immunity) was reached.

[0166] The absorbency of the serum increases as one would expect (cf. figure 7). Conversely, the effect of LA is better seen for the lower than for the higher dose used. This may be due to experimental variability or else reflect nonlinearity of dose vs. action curve for the typical immunisation data. It is possible, for example, that adjuvant is only efficient in the low dose range, whereas in the high dose regiment the system is quasi-saturated, leaving little possibility for the adjuvant to further enhance the immune response within the scope of experimental set-up. Complete animal protection against a normally lethal challenge with 50 LD_{50} was achieved in this test series with the higher TT dose in combination with LA only.

[0167] It was further observed that Th1-cytokine IgG2b was higher with LA groups, compared with the groups that received no LA. This difference was more pronounced for low doses, by the factor of 4, than for high doses, where only an enhancement by the factor of 2 was observed. Th2-cytokine IgG1 was present predominantly, except in the low dose with LA group in which IgG2b contributed comparably.

Examples 22-23:

High molecular weight adjuvant, IL-12 cytokine effect

[0168] Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-10, plus
0.01 mg IL-12 per mL immunogen suspension

[0169] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation (partially purified as described with examples 9-11)

[0170] Application area: 2 cm^2 on the upper dorsum of Swiss albino mice.

[0171] To study the effect of cytokines on results of non-invasive, epicutaneous vaccination with tetanus toxoid, a combination of monophosphoryl lipid A with 0.4 µg IL-12 per mouse was used. 80 µg of IL-12 was administered per mouse in association with Transfersomes loaded with tetanus toxoid and monophosphoryl lipid A. The details of immunisation schedule, bleeding intervals, or the final challenge with the tetanus toxin were the same as mentioned above.

[0172] The results of experimental series are illustrated in figure 8. The corroborate the conclusion that the presence of pro Th2 cytokines in the skin during the course of immunopresentation following an epicutaneous TT administration positively affects the outcome of vaccination. This is seen in serum absorbency, the specific antibody titre as well as in the test animal survival probability.

[0173] The effect discussed with examples 22-23 was verified by incorporating cytokines other than IL-12 into immunogen formulation. The results are shown in figure 9.

Examples 24-25:

High molecular weight adjuvant (IFN-γ and GM-CSF + IL-4) effect

[0174] Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-8, plus
0.05 mg IFN-γ and 0.004 mg GM-CSF and 0.004 mg IL-4 per mL immunogen suspension

[0175] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation (impure)
[0176] Application area: 2 cm^2 on the upper dorsum of Swiss albino mice.
[0177] The effect discussed with examples 22-23 was confirmed also with a different blend of cytokines. The results are shown in figure 10.

Examples 28-29:

Booster effect (maturation of immune response)

[0178] In most of previous examples, a consistent pattern was observed whenever the absorbency was measured during the time course of immunisation. The immune response increased with each boost, compared to the response obtained after primary immunisation (see Figures 3, 4, 5, 6, 7, 8). The primary response was characterised by predominance of IgM, followed by gradual appearance of IgG after the first boost and by the appearance of even grater amounts of IgG after the second boost, with a concurrent disappearance of IgM. This typical pattern of isotype signifies affinity maturation in the immune response. During the process, the average affinity of a mixture of specific antibodies increases with repeated immunisations.
[0179] Results of various epicutaneous vaccination experiments suggest that it may be advantageous to combine an invasive priming vaccination with non-invasive secondary (boost) immunisation.

Examples 30-72:

Release of cytokines from the skin in vitro by Transfersomes

[0180] Highly deformable vesicles (Transfersomes type C):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.9 mL phosphate buffer, 50 mM, pH 7.3
2.5 µL thereof

[0181] Highly deformable vesicles (Transfersomes type T):

50 mg phosphatidylcholine from soy bean (SPC)
50 mg polysorbate (Tween 80)
0.9 mL phosphate buffer, 50 mM, pH 6.5
2.5 µL thereof

[0182] Positive control A:
2.5 pL 5% sodium dodecylsulphate (SDS)
[0183] Positive control B:
100 µL lipopolysaccharide (LPS; 10^5 U/mL)
[0184] Negative control:
2.5 pL of phosphate buffered saline (PBS)
[0185] All products were tested undiluted.
[0186] Cell type: Normal human keratinocytes, forming a pluri-stratified epithelium with a compact stratum corneum were used; histology revealed strong resemblance with human epidermis in vivo.
[0187] Method: Keratinocytes were inoculated on polycarbonate filter inserts of 0.63 cm^2 in chemically defined, supplemented medium, and cultured for 17 days at the air-liquid interface.
[0188] Test measurements: given amount of each tested product was deposited with a micropipette and spread

evenly over the surface of the stratum corneum of eight reconstituted epidermis using a small sterile device. The cultures were incubated at 37 °C, 5 % CO_2 for 24 hours. Quadruplicate cultures (except for the LPS treated cells which were incubated in duplicate) were washed with 0.5 mL of PBS and incubated on 300 µL of 0.5 mg/mL MTT for 3 hours at 37 °C, 5 % CO_2.

[0189] The release of inflammatory mediators (IL1α, IL2, IL4, IL8, IL10, IFN-γ, and TNF-α) in the medium underlying the tissues was quantified using ELISA kits (R&D systems UK; Quantikine), specific for each type of immuno-modulator to be measured.

	IL1-α (pg/mL) Mean +/- SD	IL8 (pg/mL) Mean +/- SD	TNF-α (pg/mL)
Negative control (PBS, n=2)	5.1 +/- 0.5	<31	not detectable
Positive control A (SDS 5 %, n=2)	**314.2 +/- 6.1**	**147.5 +/- 32**	not detectable
Positive control B (LPS, n=1)	32.0	**5161**	**113.4**
Transfersomes C (02-05, n=2)	12.3 +/- 0.9	68.3 +/- 16.8	not detectable
Transfersomes T (TT0009/175, n=2)	11.7 +/- 1.2	50.8 +/- 14.0	not detectable
Transfersomes O (TT0017/15, n=2)	**185.5 +/- 170.1**	58.4 +/- 27.0	not detectable
The relatively big standard deviation observed with Transfersomes O can be explained by the fact that the product was difficult to spread uniformly onto the stratum corneum of the reconstructed epidermis.			

[0190] TNF-α level was increased to the level of 113.43 pg/mL when the cells were in contact with the positive controls containing LPS, which is an established immunoadjuvant.

[0191] IL8 concentration after cells incubation with Transfersomes exceeded the lower limit of detection by just the factor of 2, which in one case is not and in the other is barely significant at 95 % confidence level, but in either situation is negligible compared to the increase observed with the positive control containing the immunoadjuvant LPS, which gave a 167x higher value.

[0192] Non-specific irritant, SDS, released a great quantity of IL-1α from the skin cells into the bathing medium in vitro. The possibility exists, that an amount of comparable quantity was released from the cells incubated with Transfersomes O, comprising the potentially irritating oleic acid at a high concentration, but firm conclusion is prevented by the great standard deviation in the results obtained with the latter test system.

[0193] IL-1α concentration for the other tested Transfersomes of type A and type B changed to approximately 2 times the background level. This difference is statistically significant, compared to negative controls, but practically negligible, taken that the increase observed with the positive control containing LPS was more than 60 times higher.

[0194] IFN-γ, IL-2, IL4 or IL10 was not elevated to a measurable level, suggesting a lack of release of these cytokines, under any other test condition.

[0195] Taken together the above mentioned findings suggest that Transfersomes do not release cytokines or induce the generation of such molecules from the skin cells. This explains the need for using immunoadjuvants/modulators when antigens or allergens are to be delivered across the skin with such carriers and elicit a therapeutic or prophylactic immune response.

Examples 73-82:

Bacterial wall component, cholera toxin, as specific immuno-adjuvant:

[0196] Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera toxin (CT; Sigma, Neu-Ulm), 10 μg/immunisation plus, if required,
Tetanus toxoid (TT, pure; Accurate antibodies) 2 mg/mL,

[0197] Volume doses corresponding to 0 μg TT/mouse/immunisation (negative control), 1 μg TT/mouse, 5 μg TT/mouse, 10 μg TT/ mouse, 20 μg TT/ mouse, 40 μg TT/mouse (in the case of CT usage) and 80 μg TT/mouse (without CT) were used epicutaneously over an area of up to 2 cm^2 on the upper dorsum of 4-6 Swiss albino mice; 20 μg TT/ mouse/immunisation were injected subcutaneously at the corresponding site in the positive control group. Unimmunised mice were used as another negative control.

[0198] The protective effect of epicutaneous antigen administration was excellent when cholera toxin was included into the test formulation in combination with the tetanus toxoid. Formulation without this immunoadjuvant yielded inferior protection, as demonstrated by the fact that 1 animal out of 4 (25 %) was paralytic after the challenge with tetanus toxin.

[0199] The results shown in figure 10 reveal that the antigen doses in excess of 20 μg/immunisation ensured complete protection, which was not the case with the other tested adjuvants or adjuvant treatments (see previous examples). Lower dosage of antigen gave qualitatively similar effect but was insufficient to guarantee the survival of all test mice, except in the test group which received 5 μg TT/immunisation. (This implies that TT doses between 1 μg/immunisation and 15 μg/immunisation belong to the transition region.) Other doses of cholera toxin might be equally or even more beneficial, however.

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Claims

1. A transdermal vaccine comprising

   (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains;
   (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself; and
   (c) an antigen or an allergen.

2. The vaccine according to claim 1, wherein the compound displaying or inducing cytokine or anti-cytokine activity and the antigen are associated with the penetrant.

3. The vaccine according to any one of claims 1 or 2, wherein the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

4. The vaccine according to any one of claims 1 to 3, wherein the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.

5. The vaccine according to any one of claims 1 to 4, wherein the total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 weight-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.

6. The vaccine according to any one of claims 1 to 5, wherein total antigen concentration is between 0.001 and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.

7. The vaccine according to any one of claims 1 to 6 further comprising

   (da) a low molecular weight chemical irritant; and/or
   (db) an extract or a compound from a pathogen or a fragment or a derivative thereof.

8. The vaccine according to any one of claims 1 to 7 wherein the compound exerting cytokine activity is IL-4, IL-2, TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, preferably IFN-alpha or IFN-β, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

9. The vaccine according to any one of claims 1 to 8 wherein the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.

10. The vaccine according to any one of claims 1 to 9 wherein the antigen is derived from a pathogen.

11. The vaccine according to claim 10 wherein said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate

within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.

12. The vaccine according to any one of claims 1 to 11, wherein the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

13. The vaccine according to any of claims 1 to 12, wherein the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen dose and immunoadjuvant chosen, performed by injecting the formulation or performing the tests *in vitro,* and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

14. The vaccine according to any one of claims 7 to 13, wherein the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo- and endotoxins, preferably cholera toxin and the heat labile toxin of *E. coli,* an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

15. The vaccine according to claim 14 wherein said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.

16. The vaccine according to any one of claims 7 to 13, wherein the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

17. The vaccine according to any one of claims 7 to 16 wherein said low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines, (fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

18. The vaccine according to any one of claims 7 to 17, wherein the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or a comparable subject is deemed to be unacceptable owing to the local irritancy, as assessed by the methods and standards commonly used to test such an irritant.

19. The vaccine according to any one of claims 7 to 16 wherein the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., a part of implantation material.

20. The vaccine according to any one of claims 1 to 19, wherein the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunisation, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.

21. The vaccine according to any one of claims 1 to 20, wherein the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2}, even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2}, and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2}.

22. The vaccine according to any one of claims 1 to 21 wherein said antigen is a pure or purified antigen.

23. A kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine according to any one of claims 1 to 22.

24. The kit according to claim 23 further comprising at least one injectable dose of the antigen specified in claim 11 or of the allergen specified in claim 12.

25. Use of a vaccine according to any one of claims 1 to 22 for the preparation of a pharmaceutical composition for generating a protective immuno response in a mammal.

26. The use according to claim 25 wherein different treatment areas are selected to control the appiled immunogen dose and the outcome of therapeutic vaccination.

27. The use according to claim 25 or 26, wherein a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.

28. The use according to any one of claims 25 to 27, wherein the vaccine according to any one claims 1 to 22 is to be applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

29. The use according to any one of claims 25 to 28 wherein the immunogen is to be applied in a non-occlusive patch.

30. The use of any one of claims 25 to 29 **characterised in that** at least one dose of vaccine is to be administered.

31. The use according to claim 30 wherein said vaccine is to be administered as a booster vaccination.

32. The use according to claim 31, wherein the primary immunisation is done invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destructing method, and wherein the at least one subsequent, booster immunisation is to be done non-invasively.

33. The use according to any one of claims 25 to 32, wherein the vaccine is to be applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immunotolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.

34. The use according to claim 33, wherein the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.

35. The use according to any one of claims 25 to 34, wherein the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is to be determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

36. Use of the transdermal carrier, the compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined in any one of the preceding claims for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

Patentansprüche

1. Transdermaler Impfstoff, umfassend

   (a) einen transdermalen Träger, der ein Durchdringungsmittel ist, das in einem wäßrigen Lösungsmittel suspendiert oder dispergiert ist, in der Form eines winzigen Flüssigkeitstropfens, der von einer membranähnlichen Hülle aus einer oder mehreren Schichten von mindestens zwei verschiedenen Stoffen oder zwei verschiedenen Formen eines Stoffes, mit der Tendenz zu aggregieren umgeben ist, wobei die Stoffe oder Formen eines Stoffes sich mindestens um den Faktor 10 in der Löslichkeit in einem vorzugsweise wäßrigen, flüssigen Medium unterscheiden, so daß der mittlere Durchmesser von Homoaggregaten des löslicheren Stoffes oder Form des Stoffes oder der mittlere Durchmesser der Heteroaggregate bestehend aus beiden Stoffen oder Formen des Stoffes kleiner ist als der mittlere Durchmesser der Homoaggregate des weniger löslichen Stoffes oder Form des Stoffes, und/oder wobei die löslichere Komponente die Tendenz hat, den durchdringenden Tropfen zu solubilisieren, und wobei der Gehalt einer solchen Komponente bis zu 99 mol-% der Konzentration beträgt, die zum Solubilisieren des Tropfen erforderlich ist, oder ansonsten bis zu 99 mol-% der Sättigungskonzentration in dem nicht solubilisierten Tropfen entspricht, was auch immer höher ist, und/oder wobei die elastische Deformationsenergie des Tropfens, der die membranähnliche Hülle umgibt, mindestens fünffach niedriger ist, vorzugsweise mindestens zehnfach niedriger und idealerweise mehr als zehnfach niedriger ist als diejenige von roten Blutzellen oder von Phospholipid-Doppelschichten mit flüssigen, aliphatischen Ketten;
   (b) eine Verbindung, die spezifisch Cytokin- oder Anti-Cytokin-Aktivität freisetzt oder induziert oder selbst solch eine Aktivität zeigt; und
   (c) ein Antigen oder ein Allergen.

2. Impfstoff nach Anspruch 1, wobei die Verbindung, die Cytokin- oder Anti-Cytokin-Aktivität aufweist oder induziert, und das Antigen mit dem Durchdringungsmittel verknüpft sind.

3. Impfstoff nach einem der Ansprüche 1 oder 2, wobei das weniger lösliche, selbstaggregierende Molekül ein polares Lipid ist und die löslichere Komponente ein grenzflächenaktiver Stoff oder ein grenzflächenaktives Stoffähnliches Molekül ist oder ansonsten diejenige Form eines polaren Lipides, die genügend löslich für den Zweck der Erfindung ist.

4. Impfstoff nach einem der Ansprüche 1 bis 3, wobei der mittlere Durchmesser des Durchdringungsmittels zwischen 30 nm und 500 nm ist, vorzugsweise zwischen 40 nm und 250 nm, bevorzugt zwischen 50 nm und 200 nm und besonders bevorzugt zwischen 60 nm und 150 nm ist.

5. Impfstoff nach einem der Ansprüche 1 bis 4, wobei das Gesamtgewicht der Tropfen in der Formulierung zum Gebrauch auf menschlicher oder tierischer Haut 0,01 Gewichtsprozent bis 40 Gewichtsprozent der gesamten Masse, insbesondere zwischen 0,1 Gewichtsprozent und 30 Gewichtsprozent und vorzugsweise zwischen 5 Gewichtsprozent und 20 Gewichtsprozent ist.

6. Impfstoff nach einem der Ansprüche 1 bis 5, wobei die Gesamtantigenkonzentration zwischen 0,001 und 40 Gewichtsprozent der gesamten durchdringenden Masse ist, im besonderen zwischen 0,01 Gewichtsprozent und 30 Gewichtsprozent, besser zwischen 0,1 Gewichtsprozent und 20 Gewichtsprozent und am meisten bevorzugt zwischen 0,5 Gewichtsprozent und 10 Gewichtsprozent ist.

7. Impfstoff nach einem der Ansprüche 1 bis 6, ferner umfassend

   (da) ein chemisches Reizmittel mit niedrigem Molekulargewicht; und/oder
   (db) einen Extrakt oder eine Verbindung aus einem Pathogen oder ein Fragment oder ein Derivat davon.

8. Impfstoff nach einem der Ansprüche 1 bis 7, wobei die Cytokin-Aktivität aufweisende Verbindung IL-4, IL-2, TGF, IL-6, TNF, IL-1α und IL-1β, ein Typ 1-Interferon, vorzusweise IFN-α oder IFN-β, IL-12, IFN-γ, TNF-β, IL-5 oder IL-10 ist.

9. Impfstoff nach einem der Ansprüche 1 bis 8, wobei die Anti-Cytokin-Aktivität aufweisende Verbindung ein Anti-Cytokin-Antikörper oder das entsprechende aktive Fragment, ein Derivat oder ein Analog davon ist.

10. Impfstoff nach einem der Ansprüche 1 bis 9, wobei das Antigen von einem Pathogen stammt.

11. Impfstoff nach Anspruch 10, wobei das Pathogen ausgewählt ist aus extrazellulären Bakterien, einschließlich eiterbildenden Kokken, wie *Staphylococcus* und *Streptococcus,* gram-negativen Bakterien, wie Meningococcus und Gonococcus-Arten, *Neisseria*-Arten, gram-negativen Bakterien, einschließlich Darmorganismen wie *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* und gram-positiven Bakterien (z.B. *Bacillus pestis, BCG*), besonders anaeroben, wie die *Clostridium*-Arten, Bakterien und Viren, die innerhalb von Wirtszellen überleben und sich replizieren, umfassend Mycobakterien (z.B. *M, tuberculosis*) und *Listeria monocytogenes,* Retroviren und Adenoviren, einschließlich Hepatitisvirus, (menschlicher) Immundefizienzvirus, Herpesviren, Pocken- (Winpocken), Influenza-, Masern-, Mumps- und Polio-Viren, Cytomegalovirus, Rhinovirus, usw., und Pilzen, die innerhalb von Wirtszellen gedeihen, Parasiten, einschließlich tierischen Parasiten, wie Protozoen und Helminthen, und Ectoparasiten, wie Zecken und Milben, oder *Brucella*-Arten, einschließlich des Cholera verursachenden Agens, Haemophilus-Arten, ebenso wie Pathogenen, die Parathyphus, Pest, Tollwut, Tetanus und Röteln auslösen, und Pathogenen, die verschiedene Neoplasien, Autoimmunkrankheiten oder die mit anderen pathologischen Zuständen des tierischen oder menschlichen Körpers verwandt sind, verursachen, die nicht notwendigerweise von pathogenen Infektionen herrühren.

12. Impfstoff nach einem der Ansprüche 1 bis 11, wobei das Allergen xenogenen oder endogenen Ursprungs ist, von einem Mikroorganismus, einem Tier oder einer Pflanze stammt, oder zu der Gruppe künstlicher und/oder reizender inorganischer Stoffe gehört, oder zu solchen Teilen oder Komponenten des menschlichen Körpers gehören, die fälschlicherweise durch das Körperimmunsystem prozessiert oder dem Körperimmunsystem exponiert wurden.

13. Impfstoff nach einem der Ansprüche 1 bis 12, wobei die-Konzentration jeder verwendeten Verbindung, die Cytokin-Aktivität aufweist, bis zu 1000-fach höher gewählt ist als das Konzentrationsoptimum, das in den entsprechenden Versuchen mit der Antigendosis und dem gewählten Immunadjuvanz etabliert wurde, durchgeführt mittels Injektion der Formulierung oder durch in vitro-Versuche, und vorzugsweise bis zu 100-fach, öfters bis zu 50-fach und noch besser bis zu 20-fach höher ist.

14. Impfstoff nach einem der Ansprüche 7 bis 13, wobei der Pathogenextrakt oder die Verbindung ein Lipopolysaccharid, Cordfactor (Trehalose-Dimycolat), Muramyldipeptid, oder ein anderes (Poly)saccharid oder (Poly) peptid identisch mit oder ähnlich einem immunologisch aktiven Teil einer Membran eines Pathogens ist; ein Extrakt eines Pathogens, einschließlich bakteriellen Exo- und Endotoxinen, vorzugsweise Choleratoxin und das hitzelabile Toxin von *E. coli,* ein A-Kette-Derivat, eine Komponente mit einer ADP-ribosylierenden Aktivität, ein Peptidoglycan, ein von Clostridium stammendes Toxin, oder ein gereinigtes Proteinderivat von *M. tuberculosis,* LT-R192G, Fibronectin bindendes Protein 1 von *Streptococcus pyrogenes,* oder ein äußeres Membranprotein von Gruppe B-*Neisseria meningitidis* (GBOMP).

15. Impfstoff nach Anspruch 14, wobei das Lipopolysaccharid Lipid A oder ein Derivat ist und Modifikationen davon, wie Monophosphoryl-Lipid A, oder sein Analog, wie ein Fettderivat von Saccharose.

16. Impfstoff nach einem der Ansprüche 7 bis 13, wobei die Konzentration der Pathogenverbindung, die von einem Pathogen stammt, zwischen 10-fach niedriger und bis zu 1000-fach höher ist als diejenige, die sonst mit den entsprechenden injizierten Formulierungen unter Verwendung eines ähnlichen Antigens verwendet wird, wobei die epikutan verabreichte Immunadjuvantkonzentration sich öfter von der injizierten Immunadjuvantkonzentration durch den Faktor zwischen 0,5 und 100 unterscheidet, oder besser, durch den Faktor zwischen 1 und 50 und im besten Fall zwischen 2 und 25.

17. Impfstoff nach einem der Ansprüche 7 bis 16, wobei das Reizmittel mit niedrigem Molekulargewicht ausgewählt ist aus den Klassen von allergenen Metallionen, Säuren, Basen, reizenden Flüssigkeiten, Fettalkoholen, Fettaminen, Fettäthern, Fettsulfonaten, Fettphosphaten, usw., oder anderen geeigneten Lösemitteln oder Amphiphilen, oder aus der Gruppe von grenzflächenaktiven Stoff-ähnlichen Molekülen, oft mit die Hautdurchdringung verstärkenden Eigenschaften, sowie Derivaten oder Kombinationen davon.

18. Impfstoff nach einem der Ansprüche 7 bis 17, wobei die Konzentration des Reizmittels mit niedrigem Molekulargewicht um mindestens den Faktor 2, öfters um den Faktor 5, besser um den Faktor 10 oder mehr, unterhalb der Konzentration gewählt ist, die in unabhängigen Versuchen am selben oder an einem vergleichbaren Subjekt auf Grund der lokalen Reizung als nicht akzeptabel angesehen wird, ermittelt durch die Methoden und Standards, die gewöhnlich zur Untersuchung eines solchen Reizmittels verwendet werden.

19. Impfstoff nach einem der Ansprüche 7 bis 16, wobei das Allergen zu der Klasse von Inhalationsallergenen gehört,

einschließlich, aber nicht beschränkt auf verschiedene Pollen, Sporen, Stückchen von Tierhaar, Haut, Feder, natürlichen und synthetischen Textilien, Weizen, (Haus)-Staub, einschließlich Milben, ferner Nahrungsmittel- und Medikamentenallergene, Kontaktallergene, Injektions-, Invasions- oder Depotallergene, wie verschiedene (gastrointestinale) Würmer, Echinokokken, Trichinen, usw., ein Teil eines Implantationsmaterials.

20. Impfstoff nach einem der Ansprüche 1 bis 19, wobei die verabreichte Dosis eines Antigens sich um den Faktor 0,1 bis 100 von der Dosis, die sonst im Verfahren der Immunisierung injiziert worden wäre, unterscheidet, aber öfters im Bereich zwischen 0,5 bis 50, besser zwischen 1 und 20, und idealerweise weniger als 10-fach höher ist als diejenige, die mit einer Injektion verwendet wird.

21. Impfstoff nach einem der Ansprüche 1 bis 20, wobei die verabreichte durchdringende Dosis zwischen 0,1 mg/cm^2 und 15 mg/cm^2, öfters im Bereich 0,5 mg/cm^2 und 10 mg/cm^2, und vorzugsweise zwischen 1 mg/cm^2 und 5 mg/cm^2 ist.

22. Impfstoff nach einem der Ansprüche 1 bis 21, wobei das Antigen ein reines oder gereinigtes Antigen ist.

23. Kit, umfassend mindestens eine Dosis des Impfstoffes nach einem der Ansprüche 1 bis 22, in Flaschen- oder anderweitig verpackter Form.

24. Kit nach Anspruch 23, weiterhin umfassend mindestens eine injizierbare Dosis des in Anspruch 11 definierten Antigens oder des in Anspruch 12 definierten Allergens.

25. Verwendung eines Impfstoffes nach einem der Ansprüche 1 bis 22 für die Herstellung eines Arzneimittels zur Erzeugung einer schützenden Immunantwort in einem Säuger.

26. Verwendung nach Anspruch 25, wobei verschiedene Behandlungszonen ausgewählt werden, um die verabreichte Immunogendosis und das Ergebnis der therapeutischen Impfung zu kontrollieren.

27. Verwendung nach den Ansprüchen 25 oder 26, wobei eine Suspension von antigenfreien Durchdringungsmitteln mit dem Antigen beladen wird, um sie mit dem Antigen am Tag vor der Verabreichung zu verbinden, vorzugsweise 360 Minuten, bevorzugter 60 Minuten, und noch mehr bevorzugt 30 Minuten vor der Verabreichung der entstandenen Verbindung auf die Haut.

28. Verwendung nach einem der Ansprüche 25 bis 27, wobei der Impfstoff nach einem der Ansprüche 1 bis 22 auf die Haut verabreicht wird, nachdem das Organ durch eine Immunadjuvantmanipulation vorbehandelt worden ist, wobei die Manipulation z.B. darin besteht, daß man die Haut reibt, drückt, erwärmt, einem elektrischen oder mechanischen, z.B. Ultraschall-Feld aussetzt, usw., oder das Injizieren einer nicht immunogenen Formulierung in die Haut, mit der Maßgabe, daß jede dieser Behandlungen Immunadjuvantverbindungen aus der Haut oder anderen peripheren, immunaktiven Geweben freisetzt, oder sonst die Konzentration/Dauer der Wirkung von Antagonisten auf die gewünschte Impfung reduziert.

29. Verwendung nach einem der Ansprüche 25 bis 28, wobei das Immunogen in einem nicht-okklusiven Pflaster verabreicht wird.

30. Verwendung nach einem der Ansprüche 25 bis 29, **dadurch gekennzeichnet, daß** mindestens eine Dosis des Impfstoffes verabreicht wird.

31. Verwendung nach Anspruch 30, wobei der Impfstoff als eine Booster-Impfung verabreicht wird.

32. Verwendung nach Anspruch 31, wobei die primäre Immunisierung invasiv vorgenommen wird, typischer Weise durch subkutante Injektion oder eine andere geeignete, die Hautbarriere durchdringende/zerstörende Methode, und wobei mindestens eine folgende Booster-Immunisierung nicht invasiv vorgenommen wird.

33. Verwendung nach einem der Ansprüche 25 bis 32, wobei der Impfstoff zwischen 2 und 10, vorzugsweise zwischen 2 und 7, bevorzugt bis zu 5 und am bevorzugtesten bis zu 3 mal verabreicht wird, wenn ein nicht-allergenes Antigen verwendet wird, oder im Falle eines Allergens, so oft vorgenommen wird, wie erforderlich ist, um die gewünschte Immuntoleranz zu erreichen, festgestellt durch ein geeignetes Bestimmungsverfahren, oder sonst, den Versuch als gescheitert anzusehen.

34. Verwendung nach Anspruch 33, wobei das Zeitintervall zwischen den aufeinander folgenden Impfungen zwischen 2 Wochen und 5 Jahren gewählt ist, öfters zwischen 1 Monat und bis zu 3 Jahren, häufiger zwischen 2 Monaten und 1,5 Jahren.

35. Verwendung nach einem der Ansprüche 25 bis 34, wobei der Fluß der Durchdringungsmittel, die ein Immunogen durch die verschiedenen Poren in einem definierten Hindemis tragen, als eine Funktion einer geeigneten Antriebskraft oder eines Druckes, die oder der über das Hindernis hinweg wirken, bestimmt wird, und die Daten dann in geeigneter Weise über eine charakteristische Kurve beschrieben werden, die wiederum dazu verwendet wird, die Formulierung oder Anwendung weiter zu optimieren

36. Verwendung des transdermalen Trägers, der Verbindung, die spezifisch Cytokin- oder Anti-Cytokin-Aktivität induziert oder freisetzt oder solche Aktivität zeigt, des Antigens oder Allergens, und gegebenenfalls eines Extrakts oder einer Verbindung eines Mikroorganismus oder eines Fragments oder eines Derivats davon, und/oder eines chemischen Reizmittels mit niedrigem Molekulargewicht, gemäß der Definition in einem der vorangegangenen Ansprüche zur Herstellung eines Impfstoffes zur Induktion einer schützenden oder toleranten Immunantwort.

Revendications

1. Vaccin transdermique comprenant

   (a) un porteur transdermique qui est pénétrant, en suspension ou dispersé dans un solvant aqueux, sous la forme d'une minuscule gouttelette de liquide entourée d'un enrobage de type membranaire d'une ou plusieurs couches d'au moins deux substances différentes ou deux formes différentes d'une substance ayant tendance à s'agréger, lesdites substances ou formes d'une substance différant d'au moins un facteur 10 en terme de solubilité dans un milieu liquide, de préférence aqueux, de sorte que le diamètre moyen d'homo-agrégats de la substance ou forme de la substance plus soluble ou le diamètre moyen d'hétéro-agrégats comprenant lesdites deux substances ou formes de ladite substance est inférieur au diamètre moyen d'homo-agrégats de la substance ou forme de la substance moins soluble, et/ou dans lequel le composant plus soluble a tendance à solubiliser la gouttelette pénétrante et dans lequel la teneur d'un tel composant peut atteindre 99 % molaire de la concentration requise pour solubiliser la gouttelette ou sinon correspond jusqu'à 99 % molaire de la concentration de saturation dans la gouttelette non solubilisée, suivant le plus élevé, et/ou dans lequel l'énergie de déformation élastique de la gouttelette entourant le revêtement de type membranaire est au moins 5 fois plus faible, plus préférablement est au moins 10 fois plus faible et idéalement est plus de 10 fois plus faible que celle de globules rouges ou des bicouches de phospholipides comprenant des chaînes aliphatiques fluides ;
   (b) un composé qui libère spécifiquement ou induit spécifiquement l'activité cytokine ou anticytokine ou exerce une telle activité lui-même ; et
   (c) un antigène ou un allergène.

2. Vaccin selon la revendication 1, dans lequel le composé présentant ou induisant l'activité cytokine ou anticytokine et l'antigène sont associés au pénétrant.

3. Vaccin selon l'une quelconque des revendications 1 à 2, dans lequel la molécule auto-agrégante moins soluble est un lipide polaire et le composant plus soluble est un agent tensioactif ou une molécule de type tensioactif ou sinon une forme de lipide polaire telle qu'il soit suffisamment soluble pour les besoins de l'invention.

4. Vaccin selon l'une quelconque des revendications 1 à 3, dans lequel le diamètre moyen du pénétrant est compris entre 30 nm et 500 nm, de préférence entre 40 nm et 250 nm, encore plus préférablement entre 50 nm et 200 nm et de la manière la plus préférée entre 60 nm et 150 nm.

5. Vaccin selon l'une quelconque des revendications 1 à 4, dans lequel le poids total des gouttelettes dans la formule pour utilisation sur la peau humaine ou animale est de 0,01 % en poids (% m/m) à 40 % en poids de la masse totale, en particulier entre 0,1 % m/m et 30 % m/m et le plus préférablement entre 5% m/m et 20% m/m.

6. Vaccin selon l'une quelconque des revendications 1 à 5, dans lequel la concentration totale de l'antigène est de 0,001 et 40 % m/m de la masse totale pénétrante, en particulier entre 0,01 % m/m et 30 % m/m, plus préférablement entre 0,1 % m/m et 20 % m/m et le plus préférablement entre 0,5 % m/m et 10 % m/m.

7. Vaccin selon l'une quelconque des revendications 1 à 6 comprenant en outre

(da) un irritant chimique à bas poids moléculaire; et/ou
(db) un extrait ou un composé d'un pathogène ou un fragment ou dérivé de celui-ci.

8. Vaccin selon l'une quelconque des revendications 1 à 7, dans lequel le composé exerçant une activité cytokine est IL-4, IL-2, TGF, IL-6, TNF, IL-1α et IL-1β, un interféron de type I, de préférence IFN-α ou IFN-β, IL-12, IFN-γ, TNF-β, IL-5 ou IL-10.

9. Vaccin selon l'une quelconque des revendications 1 à 8, dans lequel le composé présentant une activité anticytokine est un anticorps anticytokine ou le fragment actif correspondant, un dérivé ou un analogue de celui-ci.

10. Vaccin selon l'une quelconque des revendications 1 à 9, dans lequel l'antigène est dérivé d'un pathogène.

11. Vaccin selon la revendication 10 dans lequel ledit pathogène est choisi parmi des bactéries extracellulaires, comprenant les cocci formant du pus, tels que *Staphylococcus* et *Streptococcus,* des bactéries gram négatif, telles que les espèces Meningococcus et Gonococcus, les espèces de *Neisseria,* des bactéries gram négatif, comprenant des organismes entériques tels que *E. coli, Salmonella, Shigella, Pseudomonas, Diphteria, Bordetella, Pertussis,* et des bactéries gram positif (par exemple, *Bacillus pestis, BCG*), en particulier des anaérobies tels que les espèces *Clostridium,* les bactéries et les virus, qui survivent et se répliquent à l'intérieur des cellules hôtes, comprenant les mycobactéries (par exemple, *M. tuberculosis*) et *Listeria monocytogenes,* des rétrovirus et adénovirus, comprenant le virus de l'hépatite (humain), le virus d'immunodéficience, les virus de l'herpès, les virus de la petite vérole (varicelle), la grippe, la rougeole, les oreillons et la polio, le cytomégalovirus, le rhinovirus, etc., et les champignons proliférant à l'intérieur de cellules hôtes, les parasites comprenant des parasites animaux, tels que les protozoaires et les helminthes, et les ectoparasites, tels que les tiques et les mites, ou les espèces de *Brucella,* comprenant l'agent causal du choléra, les espèces de Haemophilius, ainsi que des pathogènes déclenchant les maladies paratyphoïde, peste, rage, tétanos et rubéole et les pathogènes provoquant différentes néoplasies, des maladies auto-immunes ou autres associées à d'autres états pathologiques du corps animal ou humain qui ne résultent pas nécessairement d'infections pathogènes.

12. Vaccin selon l'une quelconque des revendications 1 à 11, dans lequel l'allergène est d'origine xénogène ou endogène, dérivé d'un microorganisme, un animal ou une plante, ou appartenant au groupe des substances synthétiques et/ou irritantes inorganiques, ou à des parties ou composants du corps humain qui ont été incorrectement traités par ou exposés au système immunitaire corporel.

13. Vaccin selon l'une quelconque des revendications 1 à 12, dans lequel la concentration de chaque composé présentant une activité cytokine utilisée est choisie de façon à être jusqu'à 1000 fois plus forte que la concentration optimale déterminée dans les essais correspondants avec la dose d'antigène et l'immunoadjuvant choisi, conduits en injectant la formule ou en conduisant les essais *in vitro,* et préférablement jusqu'à 100 fois, plus préférablement jusqu'à 50 fois, et encore plus préférablement jusqu'à 20 fois plus élevée.

14. Vaccin selon l'une quelconque des revendications 7 à 13, dans lequel l'extrait ou composé de pathogène est un lipopolysaccharide, le facteur médullaire dimycolate de tréhalose), un dipeptide de muramyle, ou un autre (poly) saccharide ou (poly)peptide identique à ou ressemblant à une partie immunologiquement active d'une membrane d'un pathogène ; un extrait d'un pathogène, comprenant des exo- et endotoxines bactériennes, de préférence la toxine du choléra et la toxine thermolabile de *E. coli,* un dérivé de chaîne A, un composant ayant une activité ADP-ribosylante, un peptidoglycan, une toxine clostridienne, ou un dérivé de protéine purifié de *M. tuberculosis,* LT-R192G, la protéine fixant la fibronectine I de *Streptococcus pyrogenes,* ou une protéine de membrane externe de *Neisseria meningitidis* groupe B (GBOMP).

15. Vaccin selon la revendication 14 dans lequel ledit lipopolysaccharide est le lipide A ou un dérivé et une modification de celui-ci, tel qu'un monophosphoryllipide A, ou son analogue, tel qu'un dérivé lipidique du saccharose.

16. Vaccin selon l'une quelconque des revendications 7 à 13, dans lequel la concentration du composé pathogène dérivé d'un pathogène est 10 fois plus faible à 1000 fois plus forte que celle utilisée avec les formulations injectées correspondantes utilisant un antigène similaire, la concentration d'immunoadjuvant administrée par voie épicutanée différant plus souvent de la concentration d'immunoadjuvant injectée d'un facteur compris entre 0,5 et 100, ou plus préférablement, d'un facteur compris entre 1 et 50, et le plus préférablement entre 2 et 25.

17. Vaccin selon l'une quelconque des revendications 7 à 16, dans lequel ledit irritant à bas poids moléculaire est choisi parmi les classes d'ions de métal, acides, bases, liquides irritants, alcools (gras), amines (grasses), éthers (gras), sulfonates (gras), phosphates, etc. allergènes, ou d'autres solvants ou amphiphiles adaptés, ou dans le groupe des molécules de type tensioactif, présentant souvent la capacité d'améliorer la perméation de la peau, ainsi que des dérivés ou combinaisons de ceux-ci.

18. Vaccin selon l'une quelconque des revendications 7 à 17, dans lequel la concentration d'un irritant à bas poids moléculaire est choisi pour être d'au moins un facteur 2, plus préférablement d'un facteur 5, et encore plus préférablement d'un facteur 10 ou plus, inférieure à la concentration qui, dans des essais indépendants sur un sujet identique ou comparable est jugée inacceptable en raison de l'irritation locale, comme déterminé par les méthodes et standards couramment utilisés pour tester un tel irritant.

19. Vaccin selon l'une quelconque des revendications 7 à 16, dans lequel l'allergène appartient à la classe des allergènes par inhalation, comprenant sans y être limités différentes pollens, spores, fragments de poil, peau, plumes d'animal, textiles naturels et synthétiques, blé, poussières (domestiques), comprenant les mites ; de plus, des allergènes alimentaires et pharmaceutiques ; des allergènes de contact ; des allergènes par injection, invasion ou dépôt, tels que différents vers (résidant dans le tube digestif), echinococcus, trichines, etc., un fragment de matériau d'implantation.

20. Vaccin selon l'une quelconque des revendications 1 à 19, dans lequel la dose appliquée d'un antigène diffère d'un facteur de 0,1 à 100 de la dose qui aurait dû être injectée dans le processus d'immunisation, mais plus souvent est dans l'intervalle compris entre 0,5 et 50, plus préférablement entre 1 et 20 et idéalement, est inférieur à 10 fois plus que celle qui est utilisée avec une injection.

21. Vaccin selon l'une quelconque des revendications 1 à 20, dans lequel la dose pénétrante appliquée est comprise entre 0,1 $mg.cm^{-2}$ et 15 $mg.cm^{-2}$, plus souvent dans l'intervalle de 0,5 $mg.cm^{-2}$ et 10 $mg.cm^{-2}$, et de préférence entre 1 $mg.cm^{-2}$ et 5 $mg.cm^{-2}$.

22. Vaccin selon l'une quelconque des revendications 1 à 21, dans lequel ledit antigène est un antigène pur ou purifié.

23. Kit comprenant, sous une forme flaconnée ou un autre conditionnement, au moins une dose du vaccin selon l'une quelconque des revendications 1 à 22.

24. Kit selon la revendication 23 comprenant également au moins une dose injectable de l'antigène spécifié dans la revendication 11 ou de l'allergène spécifié dans la revendication 12.

25. Utilisation d'un vaccin selon l'une quelconque des revendications 1 à 22 pour la préparation d'une composition pharmaceutique pour générer une réponse immunoprotectrice chez un mammifère.

26. Utilisation selon la revendication 25 dans laquelle différents domaines de traitement sont sélectionnés pour contrôler la dose d'immunogène appliquée et le résultat de la vaccination thérapeutique.

27. Utilisation selon la revendication 25 ou 26, dans laquelle une suspension de pénétrants sans antigène est chargée avec l'antigène pour être associée à celui-ci pendant le jour précédant l'administration, de préférence 360 min, plus préférablement 60 min et encore plus préférablement 30 min avant l'administration de la formulation résultante sur la peau.

28. Utilisation selon l'une quelconque des revendications 25 à 27, dans laquelle le vaccin selon l'une quelconque des revendications 1 à 22 doit être appliqué sur la peau après prétraitement de l'organe par une manipulation immunoadjuvante, ladite manipulation comprenant, par exemple, abrasion, pression, chauffage, exposition à un champ électrique ou mécanique, des ultrasons par exemple, etc. de la peau ou l'injection d'une formule non immunogène dans la peau, à condition qu'un tel traitement libère des composés immunoadjuvants depuis la peau ou d'autres tissus immunoactifs périphériques ou diminue autrement la concentration / durée d'action d'antagonistes de la vaccination souhaitée.

29. Utilisation selon l'une quelconque des revendications 25 à 28, dans laquelle l'immunogène doit être appliqué dans un patch non occlusif.

30. Utilisation selon l'une quelconque des revendications 25 à 29, **caractérisée en ce qu'**au moins une dose de vaccin doit être administrée.

31. Utilisation selon la revendication 30 dans laquelle ledit vaccin doit être administré comme vaccination de rappel.

32. Utilisation selon la revendication 31, dans lequel l'immunisation primaire est effectuée de manière invasive, généralement en utilisant une injection sous-cutanée ou tout autre méthode de perforation/destruction de la barrière cutanée adaptée, et dans laquelle la au moins une immunisation de rappel ultérieure doit être effectuée de manière non invasive.

33. Utilisation selon l'une quelconque des revendications 25 à 32, dans laquelle le vaccin doit être appliqué entre 2 et 10, de préférence entre 2 et 7, encore plus préférablement jusqu'à 5 et plus préférablement jusqu'à 3 fois, quand un antigène non allergène est utilisé, ou un nombre de fois tel, dans le cas d'allergènes, requis pour obtenir l'immunotolérance souhaitée, déterminé suivant une méthode d'évaluation adaptée, ou sinon pour estimer l'action comme ayant échoué.

34. Utilisation selon la revendication 33, dans laquelle l'intervalle de temps entre les vaccinations successives est choisi pour être entre 2 semaines et 5 ans, souvent entre 1 mois et jusqu'à 3 ans, plus fréquemment entre 2 mois et 1,5 ans.

35. Utilisation selon l'une quelconque des revendications 25 à 34, dans laquelle le flux de pénétrants qui transporte un immunogène par l'intermédiaire des différents pores dans une barrière bien définie doit être déterminé en fonction d'une force d'entraînement ou d'une pression adaptée exercée à travers la barrière et les données sont ensuite commodément décrites par une courbe caractéristique qui est ensuite utilisée pour optimiser plus avant la formule ou application.

36. Utilisation du porteur transdermique, du composé qui libère spécifiquement ou induit spécifiquement l'activité cytokine ou anticytokine ou exerce une telle activité lui-même, de l'antigène ou allergène, et facultativement d'un extrait ou d'un composé d'un microorganisme, ou un fragment ou dérivé de celui-ci, et/ou un irritant chimique à bas poids moléculaire comme défini dans l'une quelconque des revendications précédentes pour la préparation d'un vaccin pour induire une réponse immunitaire protectrice ou tolérogène.

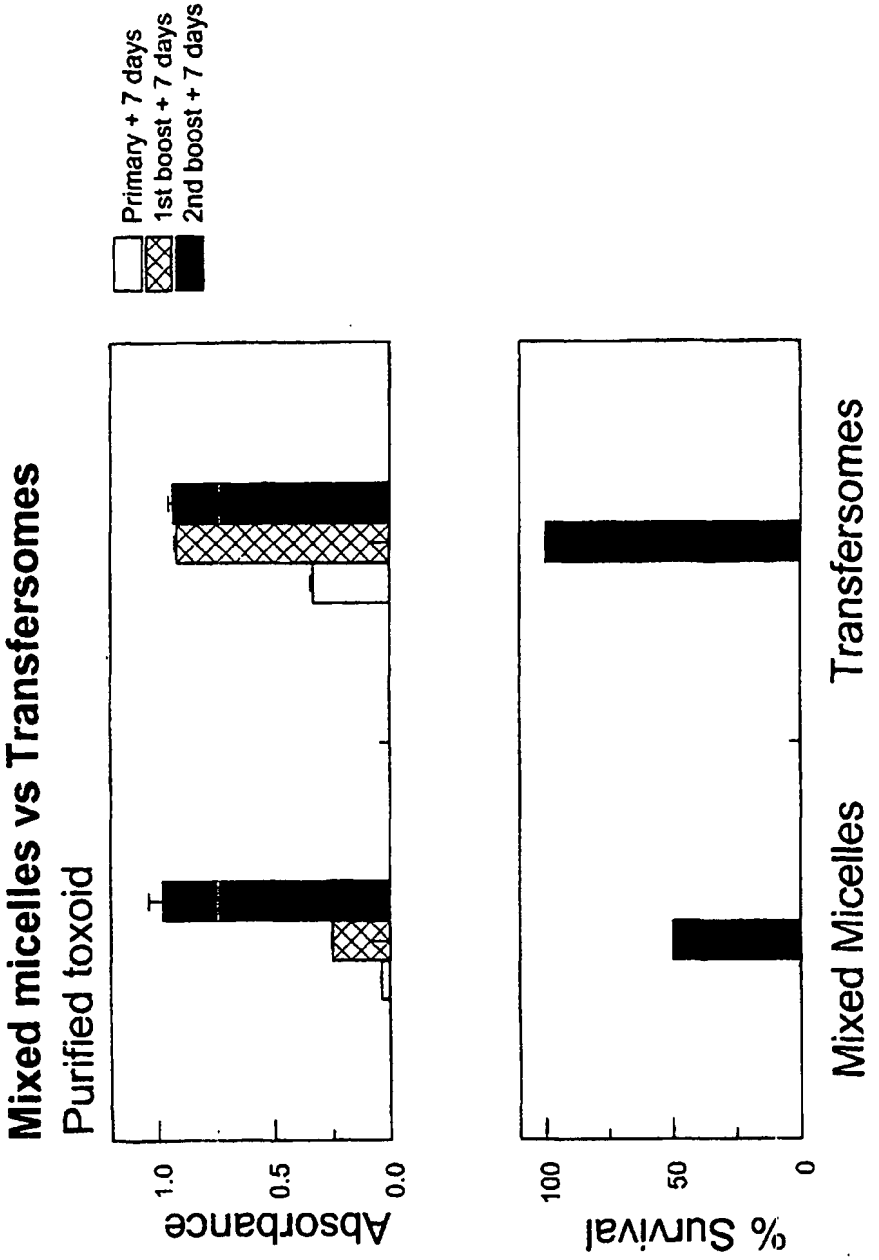

Mixed micelles vs Transfersomes
Purified toxoid
Primary + 7 days
1st boost + 7 days
2nd boost + 7 days
Absorbance
1.0
0.5
0.0
% Survival
100
50
0
Mixed Micelles
Transfersomes

Figure 1

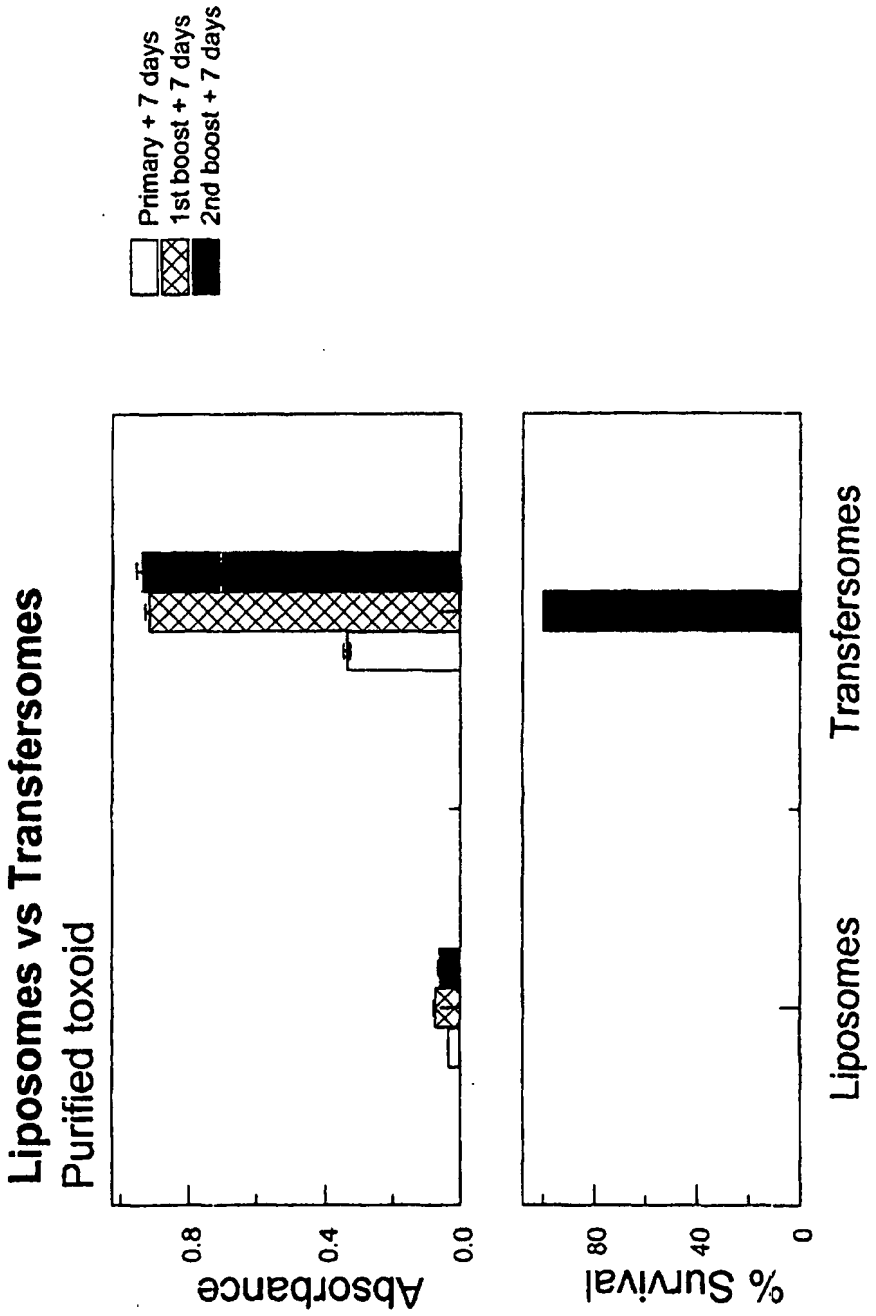

Liposomes vs Transfersomes
Purified toxoid
Primary + 7 days
1st boost + 7 days
2nd boost + 7 days
Absorbance
0.0
0.4
0.8
% Survival
0
40
80
Liposomes
Transfersomes

Figure 2

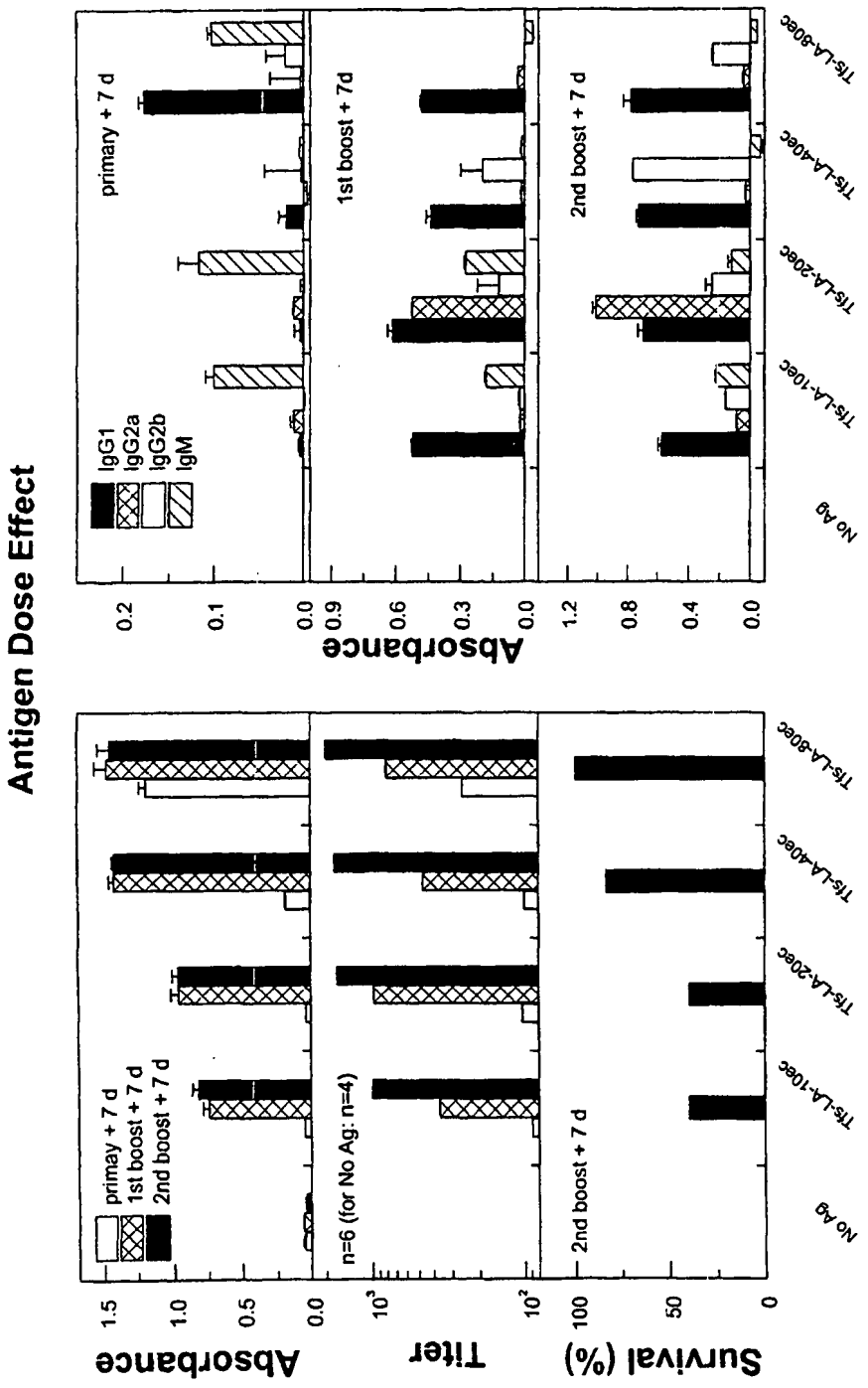

Antigen Dose Effect
Absorbance
Titer
Survival (%)
primay + 7 d
1st boost + 7 d
2nd boost + 7 d
n=6 (for No Ag: n=4)
IgG1
IgG2a
IgG2b
IgM
primary + 7 d
1st boost + 7d
2nd boost + 7 d
No Ag
Tfs-LA-10ec
Tfs-LA-20ec
Tfs-LA-40ec
Tfs-LA-80ec
antigen doses: 10, 20, 40 and 80 µg
LA: Monophosphoryl Lipid A
Tfs: Transfersomes (SPC:NaChol 3.75:1)

Figure 3

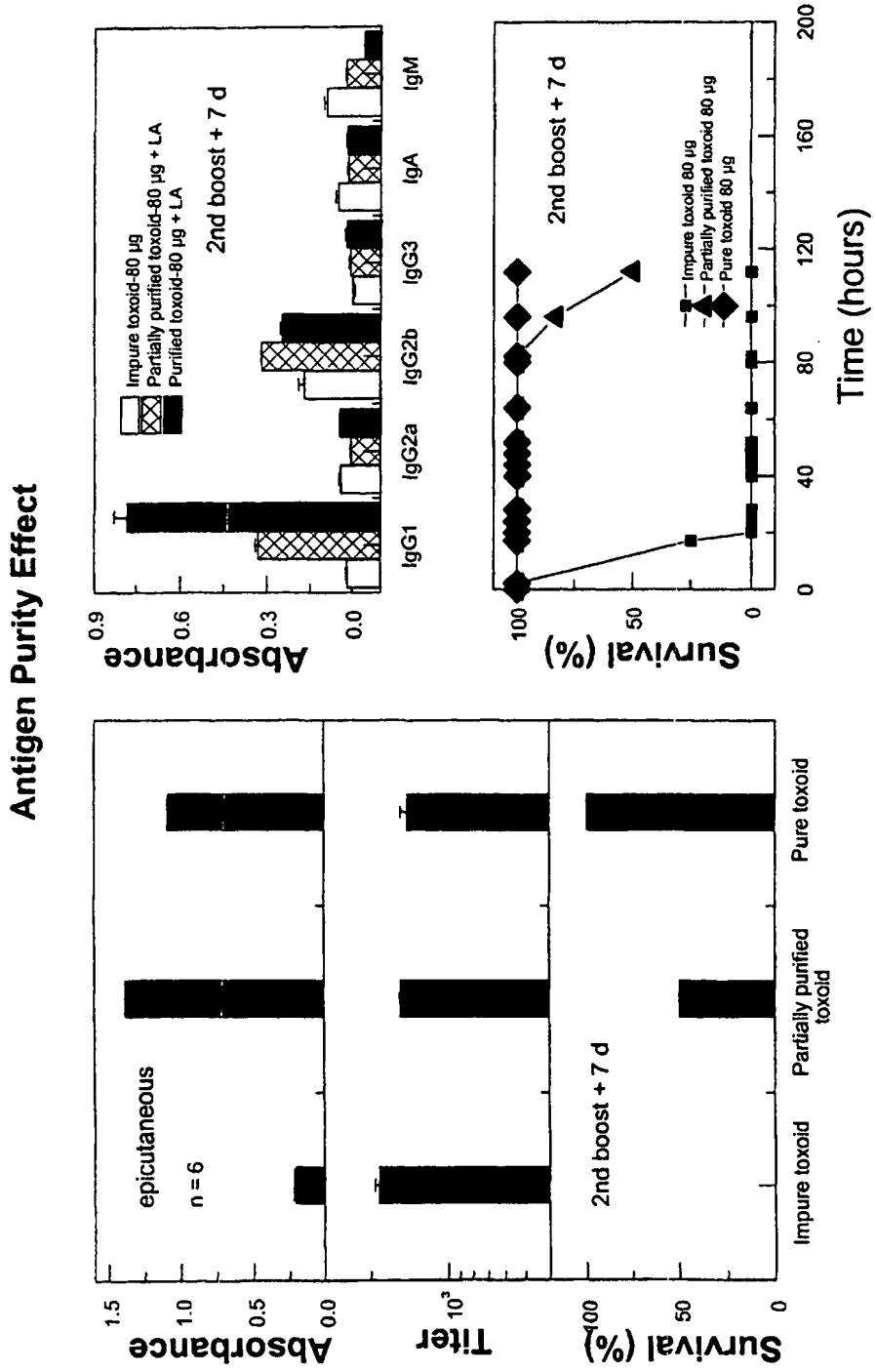


Figure 4

Immunized with Tfs (SPC:NaCh 3.75:1) containing 80 µg toxoid
LA: Monophosphoryl lipid A

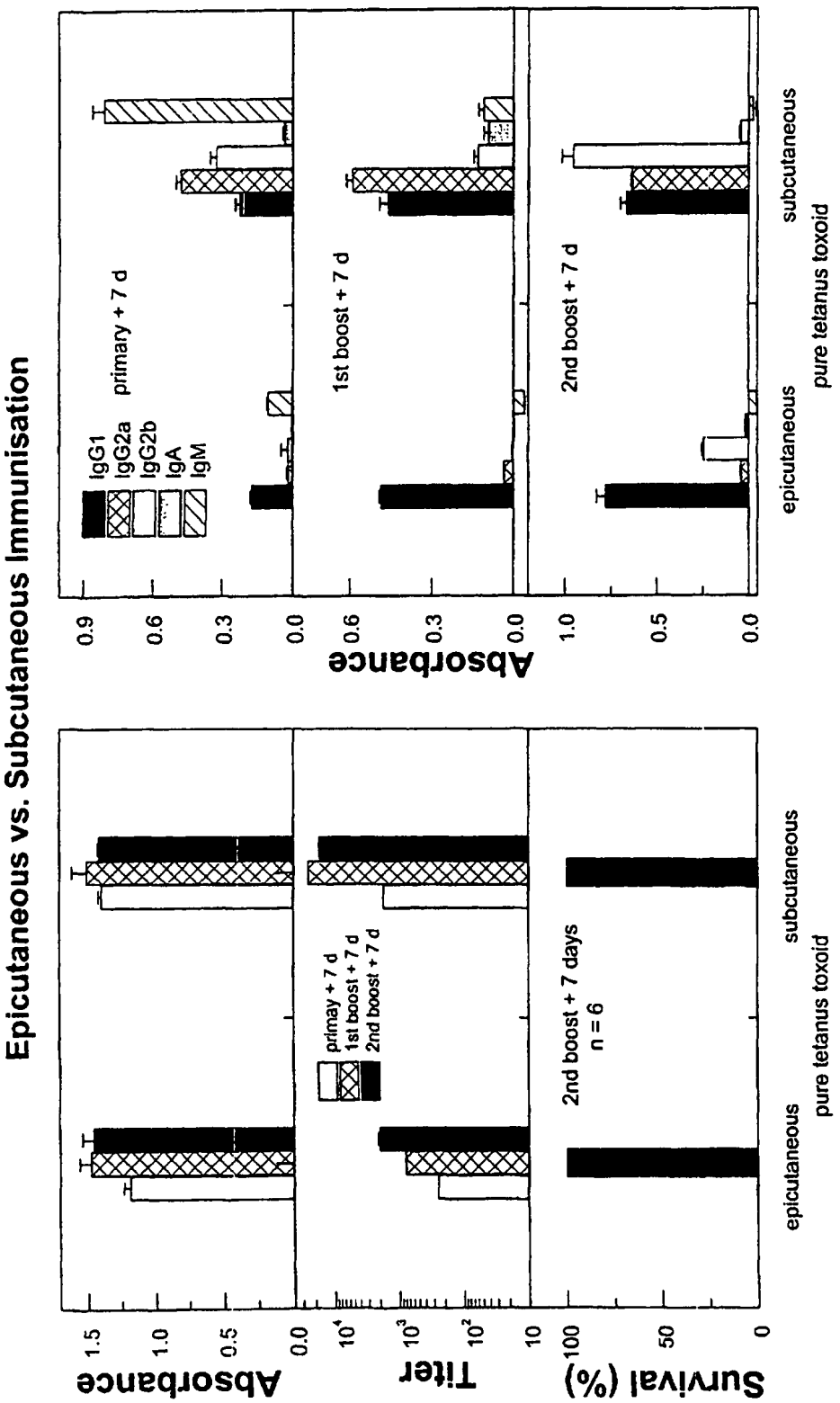

Epicutaneous vs. Subcutaneous Immunisation
Absorbance
Titer
Survival (%)
primay + 7 d
1st boost + 7 d
2nd boost + 7 d
2nd boost + 7 days
n = 6
epicutaneous
subcutaneous
pure tetanus toxoid
IgG1
IgG2a
IgG2b
IgA
IgM
primary + 7 d
1st boost + 7 d
2nd boost + 7 d

Figure 5

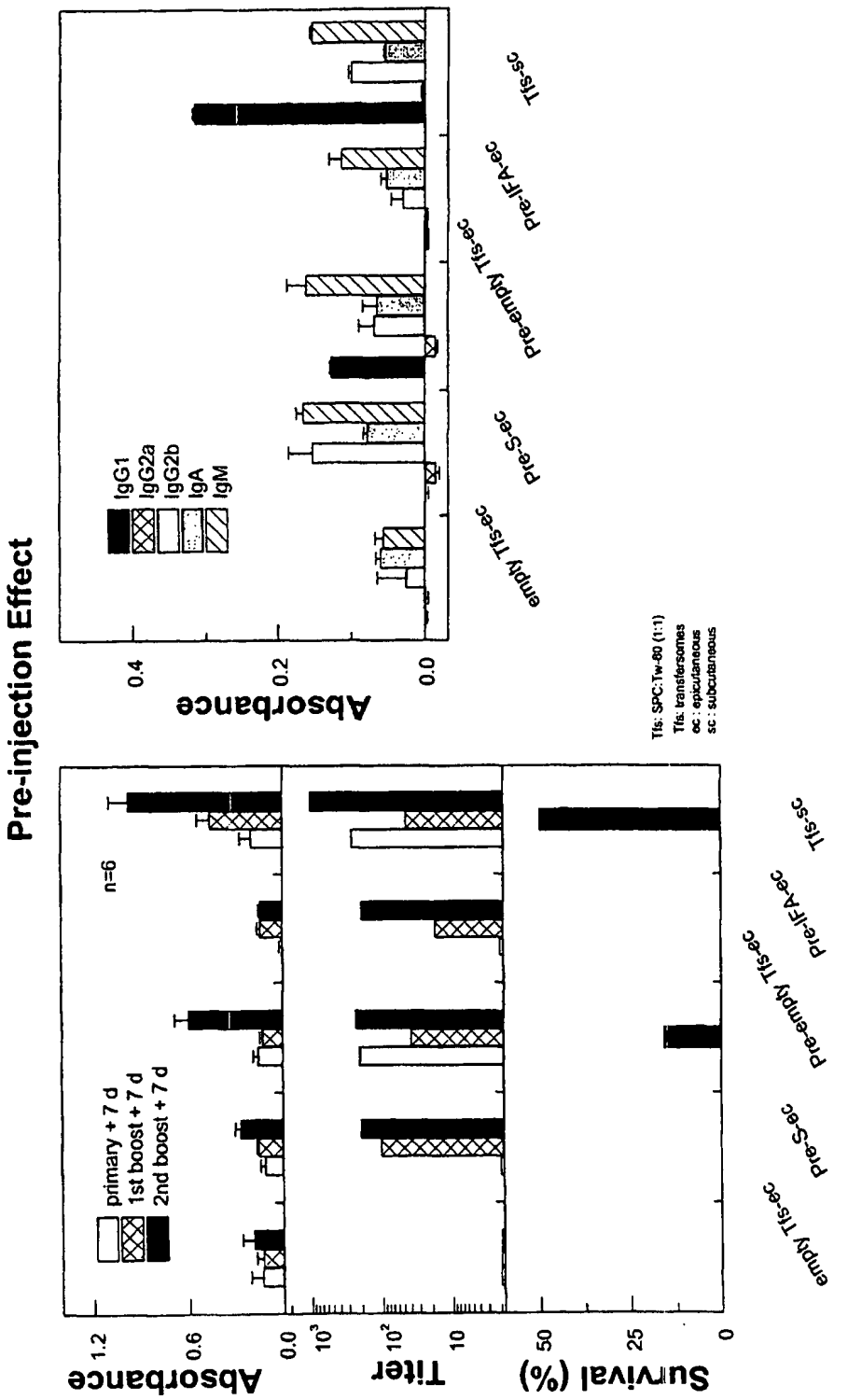


Figure 6

Mice in the preinjection groups were injected 24 hours before the application of 40 µg antigen 0.1 cc. each of saline, 10% SPC-NaCl(4:5.1) empty transfersomes and incomplete Freund's adjuvant were used for pre-injection. All the mice in this experiment were challenged with 50 times LD_{50} dose of toxin 7 days after the second boost

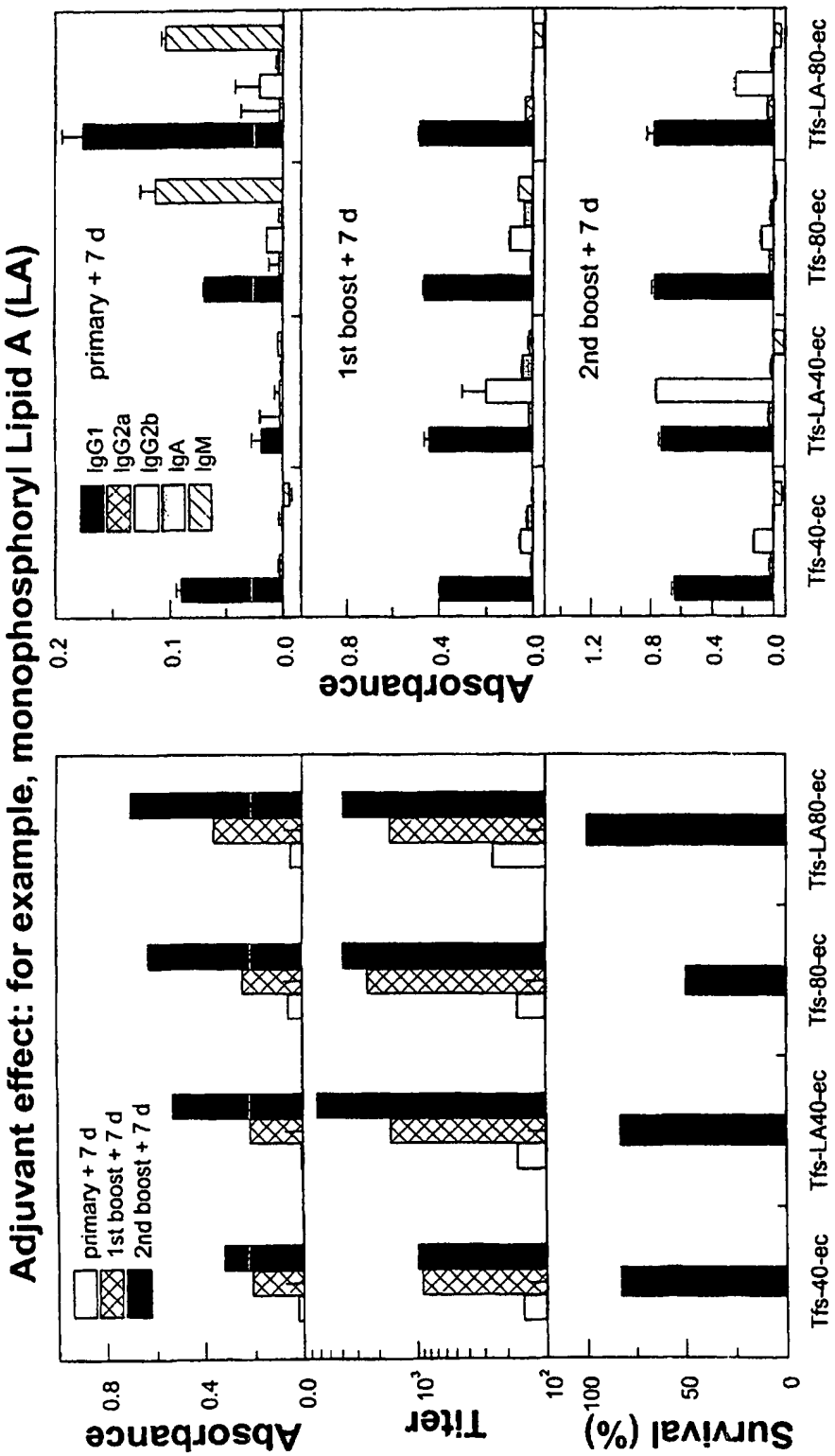

Adjuvant effect: for example, monophosphoryl Lipid A (LA)
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Absorbance
Titer
Survival (%)
Tfs-40-ec
Tfs-LA40-ec
Tfs-80-ec
Tfs-LA80-ec
* partially paralyzed but alive
IgG1
IgG2a
IgG2b
IgA
IgM
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Absorbance
Tfs-40-ec
Tfs-LA-40-ec
Tfs-80-ec
Tfs-LA-80-ec

Figure 7

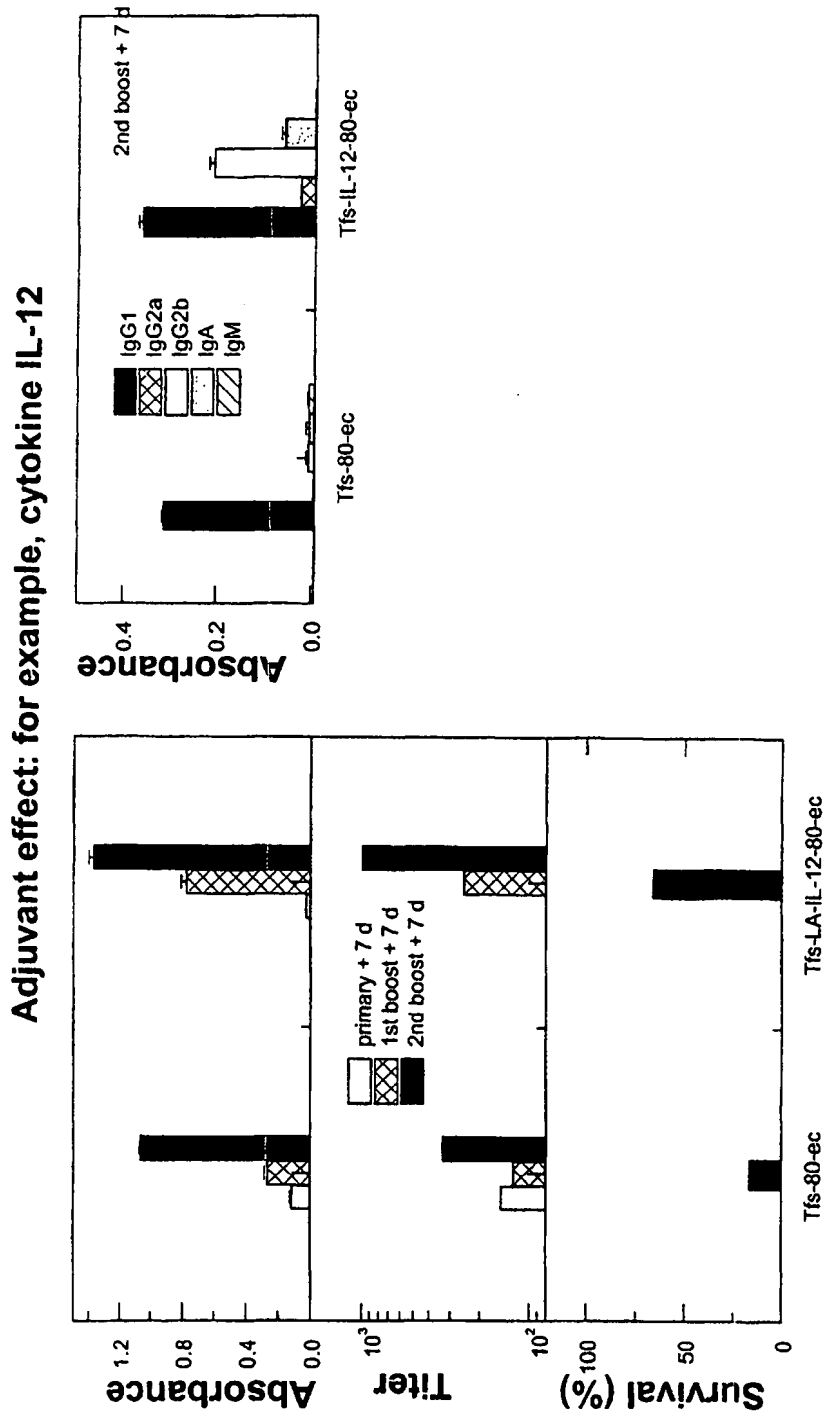

Adjuvant effect: for example, cytokine IL-12
Absorbance
Titer
Survival (%)
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Tfs-80-ec
Tfs-LA-IL-12-80-ec
IgG1
IgG2a
IgG2b
IgA
IgM
2nd boost + 7 d
Tfs-80-ec
Tfs-IL-12-80-ec
Tfs: SPC:NaCh ec: epicutaneus

Figure 8

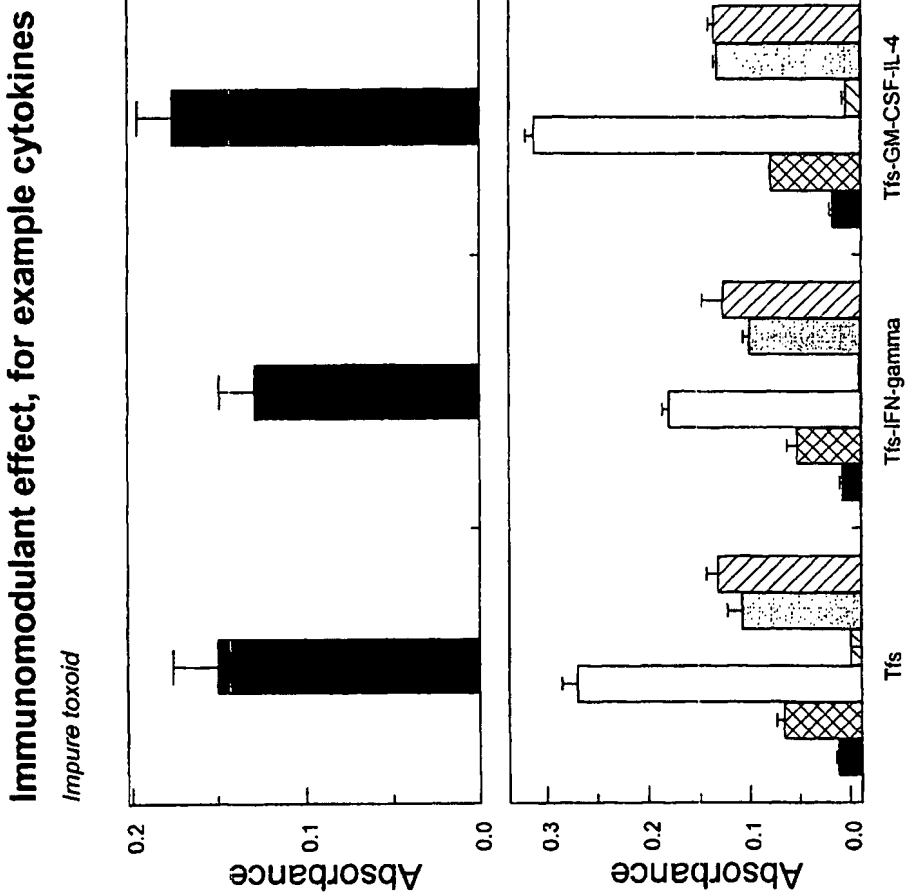
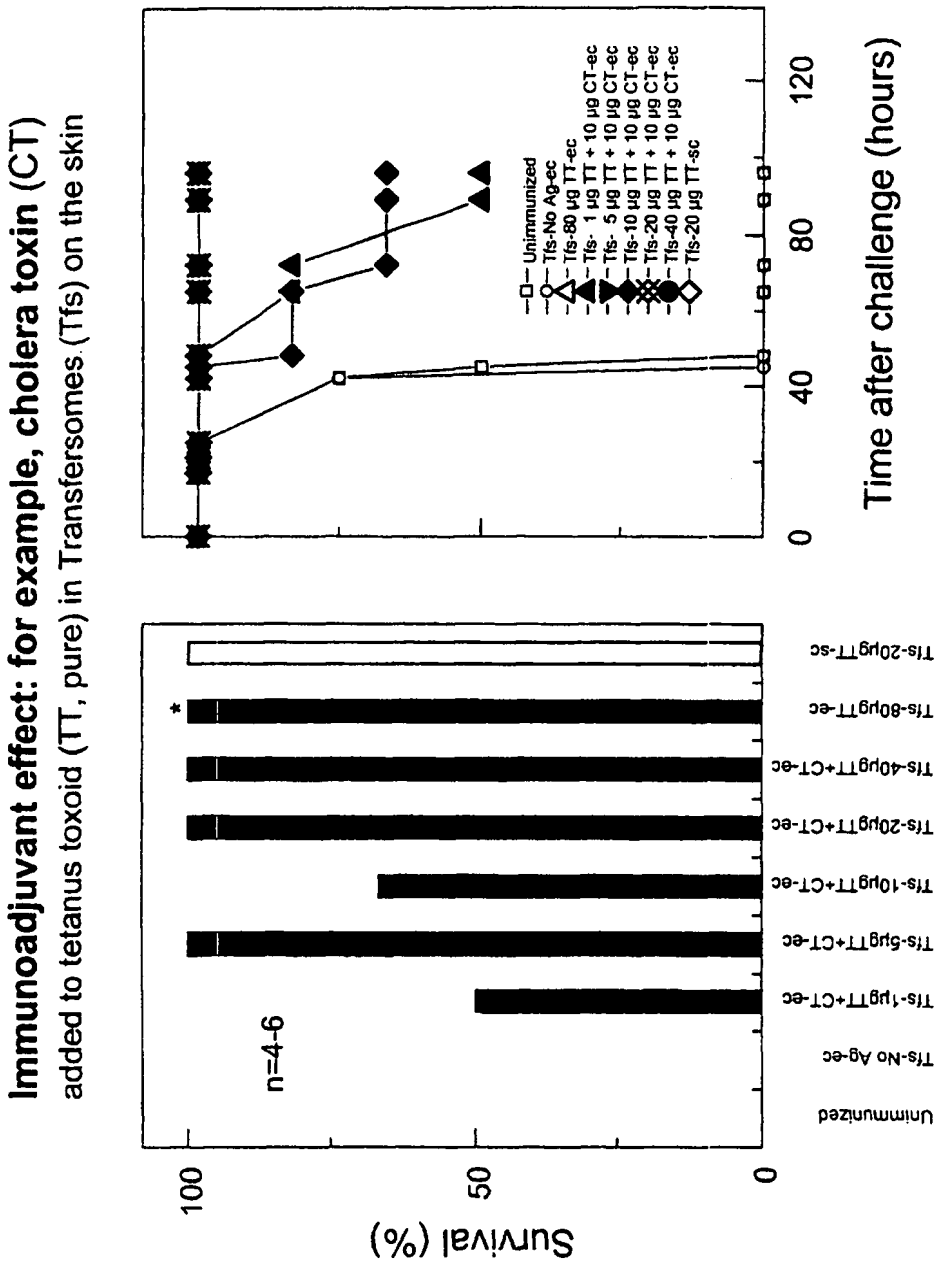


Figure 9

Serum was collected for the assay on 7th day after 2nd boost
No protection was observed in any of the groups.



Tfs: SPC:Sodium Cholate(3.75:1)
CT dose. 10 µg per mouse wherever used
n=4-6
* 1 mouse partially paralyzed out of 4

Figure 10